REMARKS

The Official Action of July 31, 2006, and the prior art relied upon therein have been carefully reviewed. The claims in the application are now claims 1-3 and 5-8, and these claims define patentable subject matter warranting their allowance. The applicants respectfully request favorable reconsideration and allowance.

Acknowledgement by the PTO of the receipt of applicants' papers filed under Section 119 is noted.

Some amendments have been introduced into the claims in order to better conform with U.S. practice. Among these is replacement of the "use" claim 4 with method of use claims 7 and 8 in accordance with U.S. practice. New claims 5-8 are patentable at least because they incorporate the subject matter of the patentable claims from which they depend.

Claims 1-4 have been rejected under the second paragraph of Section 112. The rejection is respectfully traversed.

Respectfully, the rejection appears to be misplaced. There is nothing indefinite about what is covered. The rejection appears to focus on the breadth of claim 1, rather

than what it means. In short, breadth is not indefiniteness, noting MPEP 2173.04.

Applicants believe the claims as originally drafted, particularly when considered in light of applicants' specification (fully consistent with the law), would not be confusing to those skilled in the art, and therefore the claims in their previous form are fully in accordance with Section 112. At **worst**, claim 1 in its previous and original form might possibly be considered objectionable, but only as to form, requiring no substantial amendments relating to patentability.

Nevertheless, in deference to the examiner's views and to minimize argumentation, claim 1 has been amended to specify that the salts and solvates of the specified compounds are pharmaceutically acceptable, support being found in applicants' specification at page 2, line 27 through page 4, line 10. New claim 5 is directed to preferred salts (chlorohydrate and trifluoroacetate salts) of the compounds of claim 1 (see page 4, lines 12-15 of applicants' specification). New claim 6 is directed to a pharmaceutical composition comprising the compound of claim 5.

The amendments are of a formal nature only, i.e. cosmetic amendments made to place the claims in better form for U.S. practice. The amendments are not "narrowing"

amendments because the scope of the claims has not been reduced. No limitations have been added and none are intended.

As regards claim 4, it has been replaced with new claims 7 and 8 which are consistent with U.S. practice.

Withdrawal of the rejection is in order and is respectfully requested.

Claim 4 has been rejected under Section 101 as being non-statutory. As indicated above, it has been replaced with new claims 7 and 8.

Claims 1-3 have been rejected under Section 102 as anticipated by Martin et al, Reference AD of applicants' IDS filed June 22, 2005 (Martin AD). This rejection is respectfully traversed.

Martin AD is an abstract of the full text article, reference AE of applicants' IDS filed June 22, 2005 (Martin AE). The mystery is how the abstract can disclose a compound which is not disclosed in the full text article, Martin AE. The applicants submit that the listing of this compound in Martin AD is a **typographical error**, and therefore Martin AD is not proper "prior art", noting *In re Yale*, 168 USPQ 46, 48-49 (CCPA 1970); applicants explain below in more detail. Moreover, even if the named compound were not a typographical

error, Martin AD still would not be valid prior art, because neither Martin AD nor Martin AE enable the manufacture of such compound.

Applicants now explain why Martin AD is not a valid disclosure of the claimed compound.

1/ Thus, MARTIN AD mentions the disodium salt of 4,4'-dithiobis (3-amino-1-butanesulfonic acid) as one potential inhibitor of zinc metallopeptidase activity of tetanus toxin light chain; see CAPLUS document No. 1998:505783. The CAPLUS abstract and the article of MARTIN et al (Martin AE) were both cited in the French preliminary search report issued by the European patent Office as Search Administration in connection with the French priority application FR 0303425 (enclosed as Annex 1).

As shown on the Search Report, these 2 documents are considered as related to each other and concerning the same subject-matter: according to the EPO practice both citations are linked by the symbol «&» and coded with only one code-letter (left column).

2/ The document XP-002256500 (DATABASE CAPLUS Extract), Martin AD, attached to the Preliminary Search Report, consists of three pages, or more exactly, of two pages plus one page. On the first page are mentioned:

- the title;

- the authors;
- the address;
- the full biographic reference of the article.

All these data are those of the article MARTIN et al (Martin AE), document No. XP-002092584.

Under item AB is shown the whole abstract of the article, which is identical to that of the full text article of MARTIN et al (Martin AE).

3/ Thus, the CAPLUS DATABASE Abstract No. XP-002256500 (Martin AD) discloses the abstract of the article MARTIN et al. No. XP-002092584 (Martin AE). But this abstract does not mention any disulfide compound: this is fully consistent with the whole content of the article of MARTIN et al (Martin AE) enclosed as Annex 2. This article (Martin AE) discloses monosulfide compounds as shown in tables 3 and 4, p. 3453-3454. In particular, compound 46 is the intermediate monosulfide corresponding to the claimed sulfide compound.

As indicated at p. 3458, 4th paragraph, compound 46 was prepared as previously described in the article of CHAUVEL et al. J. Med. Chem., 1994, 37, 2950-2957 (reference 26, enclosed as Annex 4). In CHAUVEL et al., compound 46 is referenced as compound 22h (see table 2, p. 2955).

There is no disclosure anywhere, in this CHAUVEL reference or in the MARTIN reference, of how to prepare a disulfide compound.

4/ The last page of the CAPLUS DATABASE Abstract No. XP-002256500 (Martin AD) then mysteriously mentions the formula of the disulfide compound under CAS Registry No. 213488-11-0. It is not apparent how the abstract XP-002256500 may refer to a Chemical Abstract Registry Number identifying a disulfide compound, **on the basis of a publication which does not disclose it**, unless a mistake has been made by the Chemical Abstract Registry Department itself when registering this compound.

Actually, it can be hypothesized that, in Tables 3 or 4 on p. 3453 and 3454 of the MARTIN et al article (Martin AE), the bond which is located on the sulur atom, and which is intended to embody the interaction of the sulfur atom with Zn• atom present on the active site of the tetanus neurotoxin, has been erroneously interpreted as meaning that the formula should be repeated twice, namely that it represents 2 units bonded through a S-S bridge.

This is clearly a contradiction to the whole content of the article of MARTIN et al (Martin AE).

When a published abstract contains a cross-reference to its original document which is contemporaneously available,

and the literal disclosure of the abstract is inconsistent with the disclosure of the original document, the abstract should be interpreted by reference to the original full text article for the purpose of ascertaining the technical reality of what has been disclosed in the abstract. When as in the present case, there is a substantial inconsistency between the original document and its abstract, it is clearly the disclosure of the original document that must prevail.

It should therefore be clear that the naming of the compound in question on the last page of Martin AD is simply an error, and thus Martin AD is not a valid reference. This is fully consistent with *In re Yale, supra*, where the Court stated in part as follows:

> It is our opinion that not only is the listing of [the compound] in [the reference] a typographical error but also this fact would be apparent to one of ordinary skill in the art when reading the [reference]. Since it is an obvious error, it cannot be said that one of ordinary skill in the art would do anything more than mentally disregard [the compound] as a misprint or mentally substitute [another compound] in its place.

The disclosure in the original document provides the strongest evidence as to what has been made available to the skilled artesian. Martin AD should therefore be disregarded on this basis alone.

Moreover, even if Martin AD were valid prior art, which it is not for the reasons pointed out above, it could still not be applied because the mentioned relied upon compound is not enabled by Martin AD, as neither Martin AD nor the fully text article Martin AE contains any disclosure which would enable the person skilled in the art to make such compound. The Court in *In re Yale, supra*, continued as follows:

> [A "chemist of ordinary skill in the art"] would not get so far in the thought process as to determine if he knew how to make [the compound], as it would have long since been discarded by him as an obvious typographical error.
>
> ***
>
> ... . The public is not put in possession of the compound... .

MPEP 2121.01, on the issue of a reference which does not contain an enabling disclosure, states in part as follows:

> The disclosure in an assertedly anticipating reference must provide an enabling disclosure of the desired subject matter; mere naming or description of the subject matter is insufficient, if it cannot be produced without undue experimentation. [citation omitted]

Applicants respectfully return to the fact that the erroneous naming of the compound relied upon in Martin AD is fully inconsistent with not only Martin AE, but also the abstract itself of Martin AD, neither of which enable the manufacture

of the misnamed compound. As stated in *In re Yale, supra*, the skilled worker, noting the inconsistency, would disregard the erroneously named compound.

Withdrawal of the rejection is in order and is respectfully requested.

The examiner has helpfully pointed out that applicants' specification does not contain the heading "Brief Description of the Drawing", and the examiner has requested correction.

Actually, applicants' specification contains no headings, e.g. no heading for the Background section, no heading for the Summary section, and no heading for the Detailed Description. However, such headings are only optional, not required. Applicants believe that no headings are necessary.

On the other hand, applicants do not object to headings, and accordingly authorize the examiner to insert headings by "Examiner's Amendment" upon allowance of the present application.

The prior art documents of record not relied upon by the PTO have been noted, along with the implication that such documents are deemed by the PTO to be insufficiently material

to warrant their application against any of applicants' claims.

Applicants believe that all issues raised in the Official Action have been addressed above in a manner that should lead to patentability of the present application. Favorable consideration and early formal allowance are respectfully requested.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant

By _____
Sheridan Neimark
Registration No. 20,520

SN:jec:kg
Telephone No.: (202) 628-5197
Facsimile No.: (202) 737-3528
G:\BN\R\RINU\Zaluski 1\Pto\2007-01-31 PCT AMD.doc

RÉPUBLIQUE FRANÇAISE

ANNEX 1

INPI
INSTITUT NATIONAL DE LA PROPRIETE INDUSTRIELLE

RAPPORT DE RECHERCHE PRÉLIMINAIRE

établi sur la base des dernières revendications déposées avant le commencement de la recherche

N° d'enregistrement national

FA 632604
FR 0303425

DOCUMENTS CONSIDÉRÉS COMME PERTINENTS		Revendication(s) concernée(s)	Classement attribué à l'invention par l'INPI
Catégorie	Citation du document avec indication, en cas de besoin, des parties pertinentes		
X	DATABASE CAPLUS AMERICAN CHEMICAL SOCIETY; 1998, XP002256500 extrait de STN Database accession no. 1998:505738 * RN 213488-11-0 * * abrégé * -& L. MARTIN ET AL: J. MED. CHEM., vol. 41, no. 18, 1998, pages 3450-3460; XP0002092584	1	C07C309/14 A61K31/185 A61P9/12
A	WO 99 36066 A (INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE ET AL) 22 juillet 1999 (1999-07-22) * le document en entier *	1-4	

DOMAINES TECHNIQUES RECHERCHÉS (Int.Cl.7)

C07C

Date d'achèvement de la recherche	Examinateur
9 octobre 2003	Van Amsterdam, L.

CATÉGORIE DES DOCUMENTS CITÉS

X : particulièrement pertinent à lui seul
Y : particulièrement pertinent en combinaison avec un autre document de la même catégorie
A : arrière-plan technologique
O : divulgation non-écrite
P : document intercalaire

T : théorie ou principe à la base de l'invention
E : document de brevet bénéficiant d'une date antérieure à la date de dépôt et qui n'a été publié qu'à cette date de dépôt ou qu'à une date postérieure.
D : cité dans la demande
L : cité pour d'autres raisons

& : membre de la même famille, document correspondant

EPO FORM 1503 12.99 (P04C14)

ANNEX 2

XP-002256500

P.D. 60:00-bmk
P. 1-3 (3)

L2 ANSWER 1 OF 1 CAPLUS COPYRIGHT 2003 ACS on STN
AN 1998:505738 CAPLUS
DN 129:254345
TI .beta.-Amino-thiols Inhibit the Zinc Metallopeptidase Activity of Tetanus Toxin Light Chain
AU Martin, Loiec; Cornille, Fabrice; Coric, Pascale; Roques, Bernard P.; Fournie-Zaluski, Marie-Claude
CS Departement de Pharmacochimie Moleculaire et Structurale, UFR des Sciences Pharmaceutiques et Biologiques, Paris, 75270, Fr.
SO Journal of Medicinal Chemistry (1998), 41(18), 3450-3460
CODEN: JMCMAR; ISSN: 0022-2623
PB American Chemical Society
DT Journal
LA English
CC 1-3 (Pharmacology)
Section cross-reference(s): 4, 10, 23, 25, 34
OS CASREACT 129:254345

AB Tetanus neurotoxin is a 150-kDa protein produced by Clostridium tetani, which causes the lethal spastic paralytic syndromes of tetanus by blocking inhibitory neurotransmitter release at central synapses. The toxin light chain (50 kDa) has a zinc endopeptidase activity specific for synaptobrevin, an essential component of the neuroexocytosis app. Previous unsuccessful attempts to block the proteolytic activity of this neurotoxin with well-known inhibitors of other zinc proteases led the authors to study the design of specific inhibitors as a possible drug therapy to prevent the progressive evolution of tetanus following infection. Starting from the synaptobrevin sequence at the level of the cleavage site by tetanus neurotoxin (Gln76-Phe77), a thiol analog of glutamine demonstrated inhibitory activities in the millimolar range. A structure-activity relation performed with this compd. led the authors to det. the requirement for the correct positioning of the thiol group, the primary amino group, and a carboxamide or sulfonamide group on the side chain. This resulted in the design of a .beta.-amino-(4-sulfamoylphenyl)glycine-thiol, the first significantly efficient inhibitor of tetanus neurotoxin with a Ki value of 35 .mu.M.

ST amino thiol zinc metallopeptidase tetanus toxin
IT Structure-activity relationship
(enzyme-inhibiting, zinc metallopeptidase-inhibiting; .beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)
IT Enzyme kinetics
(of inhibition; .beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)
IT Toxins
RL: ADV (Adverse effect, including toxicity); BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(tetanus; .beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)
IT 7326-77-4P 213487-85-5P 213487-91-3P 213487-92-4P 213487-93-5P
213487-94-6P 213487-95-7P 213487-96-8P 213487-97-9P 213487-98-0P
213487-99-1P 213488-01-8P 213488-02-9P 213488-03-0P 213488-04-1P

24.09.2003 03:40:20

213488-05-2P 213488-16-5P 213488-17-6P 213488-18-7P 213488-19-8P
213488-20-1P 213488-21-2P 213488-22-3P
RL: RCT (Reactant); SPN (Synthetic preparation); PREP (Preparation); RACT (Reactant or reagent)
(intermediate; .beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)

IT 75-64-9, tert-Butylamine, reactions 2196-57-8 2687-43-6, Benzyloxyamine hydrochloride 4025-64-3 91702-98-6 132388-69-3
213488-24-5
RL: RCT (Reactant); RACT (Reactant or reagent)
(reactant; .beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)

IT 81669-70-7
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(zinc-contg.; .beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)

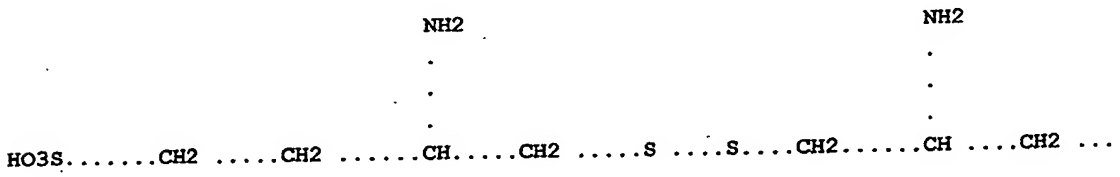
IT 213487-87-7P
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); RCT (Reactant); SPN (Synthetic preparation); THU (Therapeutic use); BIOL (Biological study); PREP (Preparation); RACT (Reactant or reagent); USES (Uses)
(.beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)

IT 213488-14-3P 213488-15-4P
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); SPN (Synthetic preparation); BIOL (Biological study); PREP (Preparation)
(.beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)

IT 141437-87-8P 162854-47-9P 213487-74-2P 213487-75-3P 213487-76-4P
213487-77-5P 213487-78-6P 213487-79-7P 213487-80-0P 213487-81-1P
213487-82-2P 213487-83-3P 213487-84-4P 213487-86-6P 213487-88-8P
213487-89-9P 213487-90-2P 213488-00-7P 213488-06-3P 213488-23-4P
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); SPN (Synthetic preparation); THU (Therapeutic use); BIOL (Biological study); PREP (Preparation); USES (Uses)
(.beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)

IT 97846-38-3 146861-98-5 156143-32-7 156143-38-3 156143-44-1
156143-66-7 156143-84-9 156144-06-8 156144-15-9 162954-89-4
213488-07-4 213488-08-5 213488-09-6 213488-10-9 ***213488-11-0***
213488-12-1 213488-13-2
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(.beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)

IT ***213488-11-0***
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(.beta.-amino-thiols inhibit zinc metallopeptidase activity of tetanus toxin light chain)
RN 213488-11-0 CAPLUS
CN 1-Butanesulfonic acid, 4,4'-dithiobis[3-amino-, disodium salt (9CI) (CA INDEX NAME)



@ 2 Na

Page 1-A
...CH2.....SO3H

Page 1-B

ANNEX 3

  J. Med. Chem. 1998, 41, 3450−3460

β-Amino-thiols Inhibit the Zinc Metallopeptidase Activity of Tetanus Toxin Light Chain

Loïc Martin, Fabrice Cornille, Pascale Coric, Bernard P. Roques, and Marie-Claude Fournié-Zaluski*

Département de Pharmacochimie Moléculaire et Structurale, U266 INSERM, URA D1500 CNRS, UFR des Sciences Pharmaceutiques et Biologiques, 4 avenue de l'Observatoire, 75270 Paris Cedex 06, France

Received March 23, 1998

Tetanus neurotoxin is a 150-kDa protein produced by *Clostridium tetani*, which causes the lethal spastic paralytic syndromes of tetanus by blocking inhibitory neurotransmitter release at central synapses. The toxin light chain (50 kDa) has a zinc endopeptidase activity specific for synaptobrevin, an essential component of the neuroexocytosis apparatus. Previous unsuccessful attempts to block the proteolytic activity of this neurotoxin with well-known inhibitors of other zinc proteases led us to study the design of specific inhibitors as a possible drug therapy to prevent the progressive evolution of tetanus following infection. Starting from the synaptobrevin sequence at the level of the cleavage site by tetanus neurotoxin (Gln^{76}-Phe^{77}), a thiol analogue of glutamine demonstrated inhibitory activities in the millimolar range. A structure−activity relationship performed with this compound led us to determine the requirement for the correct positioning of the thiol group, the primary amino group, and a carboxamide or sulfonamide group on the side chain. This resulted in the design of a β-amino-(4-sulfamoylphenyl)glycine-thiol, the first significantly efficient inhibitor of tetanus neurotoxin with a K_i value of 35 ± 5 μM.

Introduction

Tetanus neurotoxin (TeNt) is a 150-kDa protein produced by the anaerobic bacillus *Clostridium tetani* which blocks the release of inhibitory neurotransmitter in central synapses leading to a lethal spastic paralysis.[1] TeNt is a member of the clostridial neurotoxin family, including seven botulinum neurotoxins responsible for the flaccid paralysis of botulism.[2] TeNt is composed of a heavy chain of 100 kDa linked by a disulfide bridge to a light chain of 50 kDa. The heavy chain ensures the binding, internalization, and retrograde axonal transport of the light chain into the neuronal cytosol.[3] The light chain possesses proteolytic activity and cleaves specifically VAMP/synaptobrevin,[4] an integral membrane protein of small synaptic vesicles,[5−7] which plays a critical role in the neuroexocytosis apparatus.[8−10] TeNt belongs to the M_{27} family of Zn^{2+}-metallopeptidases[11] which contains the HEXXH consensus sequence, found in the majority of zinc endopeptidases, where His^{233} and His^{237} are involved in zinc chelation and Glu^{234} in the catalytic process.[12−15] Abolition of any enzymatic activity by double mutations of Glu^{270}-Glu^{271} suggests that one of these glutamates, highly conserved among all the members of this family, could be the third zinc ligand.[15] A possible role of Tyr^{243} as an additional ligand,[16] like in the family of metzincins,[17] has also been proposed. The proteolytic activity of tetanus neurotoxin has been shown to be directed toward synaptobrevin only at its Gln^{76}-Phe^{77} peptide bond.[4] Such a narrow specificity, not common for metalloproteases, has recently been explained by an allosteric-like mechanism for TeNt. Indeed, the binding of both an acidic (S 27−55) and a basic (S 82−93) domain of synaptobrevin to tetanus toxin "exosites" is required to induce the conformational change switching on its proteolytic activity[18,19] (Figure 1A).

At the present time, there is no effective drug therapy to prevent the progressive evolution of tetanus or botulism following intoxication or infection. For these reasons, we identified the inhibition of the proteolytic activity of tetanus neurotoxin as a possible strategy for treatment following toxin exposure. Potent and selective inhibitors against TeNt proteolytic activity have yet to be obtained. Strong chelating agents specific for divalent metallic cation like EDTA or 1,10-*o*-phenanthroline[20−22] give a weak protection against this toxin at millimolar concentrations. Potent blockers of zinc peptidases such as captopril, thiorphan, and phosphoramidon have no inhibitory activity in vitro[21,23] or ex vivo.[22] Moreover, various synaptobrevin-derived peptides spanning the sequence surrounding the scissible bond failed to antagonize tetanus neurotoxin proteolytic activity even when tested at concentrations up to 1 mM.[4,21]

With the aim of designing the first selective inhibitors of TeNt, we began this study using the only available clue concerning the preference of the catalytic site: the synaptobrevin sequence at the cleavage site (QAGASQ/FETSA) (Figure 1B). Starting with synaptobrevin-derived peptides containing a thiol group as a zinc ligand, a significant inhibition in the 250 μM range was obtained. An extended structure−activity relationship analysis on this compound revealed the requirement and the position of a primary amino group, the nature of the zinc chelating group, and the nature and the length of the amino-thiol side chain. Finally, a β-amino-phenylglycine-thiol substituted in the meta position by a sulfonamide group was synthesized and shown to inhibit TeNt with a K_i value of 35 μM. This molecule

* To whom correspondence should be addressed. Tel: (33)1-43-25-50-45. Fax: (33)1-43-26-69-18.

S0022-2623(98)01015-2 CCC: $15.00
Published on Web 08/11/1998.

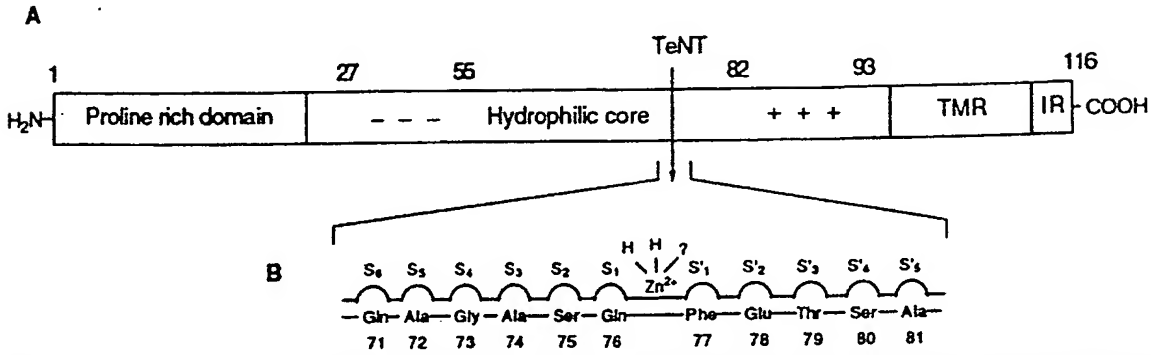


Figure 1. (A) Schematic representation of VAMP/synaptobrevin II. TMR indicates the transmembrane region and IR, the intravesicular region. The cleavage site Gln^{76}-Phe^{77} is indicated by the arrow. Domains 27–55 and 82–93 represent respectively the acidic and basic clusters whose binding to tetanus neurotoxin is required to switch on its proteolytic activity. (B) Human synaptobrevin II sequence surrounding the scissible bond. The side chains of the different synaptobrevin residues are putatively interacting with subsites of the catalytic domain of TeNt designated S_1–S_6 at the left of the cleavage bond and S'_1–S'_5 at the right, respectively.

is the first described relatively potent inhibitor of tetanus neurotoxin.

Results

Chemistry. The various thiol-containing pseudopeptides **1**–**5** spanning the putative S'_1–S'_5 subsites of TeNt active site (Table 1A) were synthesized by coupling the different tetrapeptides with the racemic (2*SR*)-3-(acetylsulfanyl)-2-benzylpropanoic acid as described in the Experimental Section.

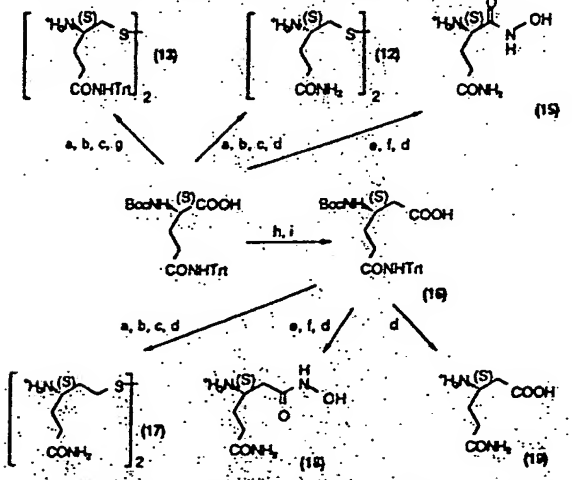
The thiol derivatives **6**–**12** (Table 1B) and **14** (Table 2) encompassing the putative subsites defined as S_1–S_5 of TeNt active domain were obtained as disulfides by coupling various protected amino acids or peptides with the β-glutamine-thiol synthon **13** as described in the Experimental Section. The synthesis of **13** (Scheme 1) was achieved by using the commercially available Boc-L-Gln(Trt)-OH. After reduction of the α-carboxylate,[24] a nucleophilic substitution of the hydroxy group by potassium thioacetate (via the Mitsunobu reaction)[25] gave a thioester group which was deprotected in alkaline conditions. A selective deprotection of the Boc group led to the synthon **13**, whereas β-amino-glutamine-thiol **12** was formed by cleavage of both the Boc and trityl groups with trifluoroacetic acid.

The different compounds described in Table 2 represent derivatives of glutamine or glutamate bearing various zinc-coordinating entities (SH, COOH, CONHOH, or PO_3H_2). Compounds **12**–**19** were synthesized following the protocol shown in Scheme 1. Compounds **37**–**39** were prepared as previously described.[26-28] The hydroxamate derivative **15** (Table 2) was synthesized from Boc-L-Gln(Trt)-OH by coupling benzyloxyamine and subsequent deprotection. Compounds **17** and **18** were obtained by the same route as **12** and **15** (Scheme 1) using the intermediate compound **16**, which has been obtained previously by Arndt–Eistert homologation of Boc-L-Gln(Trt)-OH.[29]

Compounds **40**–**53** (Table 3), **54**, and **55** (Table 4) were prepared as previously described.[26,27,30,31]

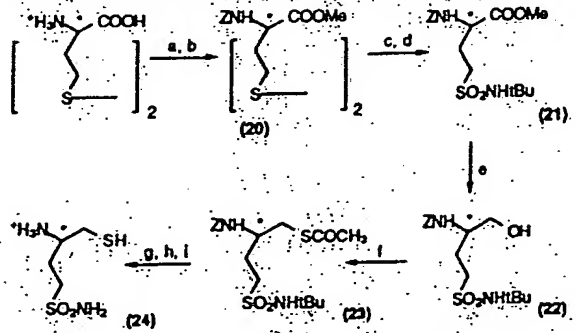
Scheme 2 depicts the synthesis of compound **24** (Table 4), the sulfonamide analogue of the β-glutamine-thiol derivative. The disulfide group of the N- and C-protected DL-homocysteine **20** was oxidized by chlorine

Scheme 1. Synthesis of the Glutamine Derivatives[a]



[a] (a) NMM, ^{t}BuOCOCl, $NaBH_4$; (b) PPh_3, (^{i}PrN=)$_2$, CH_3COSH; (c) NaOH, I_2; (d) TFA; (e) BzlONH$_2$, BOP, DIEA; (f) H_2, Pd/C; (g) $(C_2H_5)_2O{\cdot}BF_3$, AcOH; (h) NMM, ^{t}BuOCOCl, CH_2N_2; (i) Ag_2O, Na_2CO_3, $Na_2S_2O_3$, H_2O.

Scheme 2. Synthesis of the Sulfonamide Derivative[a]

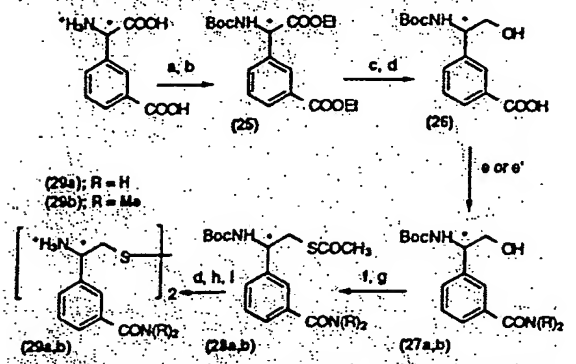


[a] (a) BzlOCOCl, NaOH; (b) CH_2N_2; (c) Cl_2, MeOH, CCl_4; (d) ^{t}BuNH$_2$; (e) $NaBH_4$; (f) PPh_3, (^{i}PrN=)$_2$, CH_3COSH; (g) NaOH; (h) TFA; (i) HF.

to give the sulfonyl chloride,[32] which was subsequently treated with *tert*-butylamine to form the *tert*-butylsulfonamide **21**. The carboxylic ester of **21** was therefore transformed into the corresponding thiol derivative **24** via the alcohol **22** substituted by potassium thioacetate to give **23** and fully deprotected by saponification and cleavage with trifluoroacetic acid and anhydrous hydrogen fluoride in succession.

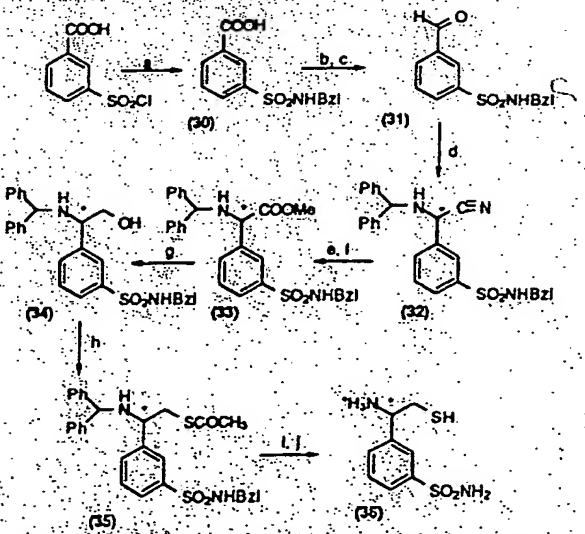
Scheme 3 shows the pathway used for the preparation of the *m*-carbamoyl analogues of *β*-phenylglycine-thiol. Starting from 3-cyanobenzaldehyde, the Bucherer–Berg reaction[33] gave the α-amino acid, which was protected to give **25**. The α-carboxylic ester was reduced to the corresponding alcohol, whereas the aryl ester, after saponification, was transformed to the carboxamide derivatives **27a,b**. The replacement of the hydroxy group by potassium thioacetate led to the thiol inhibitors **29a,b** as described above.

Scheme 3. Synthesis of the *β*-Amino-(4-carbamoylphenyl)glycine Derivatives[a]



[a] (a) $SOCl_2$, EtOH; (b) $(Boc)_2O$, NEt_3; (c) $NaBH_4$; (d) NaOH; (e) NMM, $^tBuOCOCl$, NH_3; (e′) NMM, $^tBuOCOCl$, $(CH_3)_2NH_2$; (f) CH_3SO_2Cl, NEt_3; (g) CH_3COSK; (h) I_2; (i) HCl.

Scheme 4. Synthesis of the *β*-Amino-(4-sulfamoylphenyl)glycine Derivative[a]



[a] (a) $BzlNH_2$; (b) NMM, $^tBuOCOCl$, $NaBH_4$; (c) DMSO, $(COCl)_2$, NEt_3; (d) $(Ph)_2CHNH_2$, KCN, AcOH; (e) HCl, MeOH; (f) Amberlite 120, MeOH; (g) $NaBH_4$, LiCl; (h) PPh_3, $(^iPrN{=})_2$, CH_3COSH; (i) NaOH; (j) HF.

For the synthesis of the (3-sulfamoylphenyl)glycine inhibitor **36** (Scheme 4), the commercially available 3-(chlorosulfonyl)benzoic acid was transformed into the benzylsulfonamide analogue; subsequent reduction of carboxylate led to the corresponding benzaldehyde **31**. A modified Strecker reaction allowed compound **32** to be obtained. This compound, treated successively with HCl-saturated methanol and Amberlite IR-120 in refluxing methanol, yielded the desired amino ester **33**. The subsequent steps of the synthesis, similar to those described in Scheme 2, gave the corresponding *β*-aminothiol **36**.

Inhibitory Properties. Enzymatic studies were performed using the fluorescent synaptobrevin derivative [Pya[88]]S 39-88 as substrate, according to the protocol described by Solheihac et al.[34] The various pseudopeptides **1–11** (Table 1A,B), derived from synaptobrevin sequences at the cleavage site, did not inhibit or gave weak inhibition of TeNt activity at 1 mM, whereas the *β*-amino-glutamine-thiol **12**, which is hypothesized to interact with the S_1 subsite, completely inhibited the peptidase action of TeNt at this concentration.

Table 1. Exploration of the Catalytic Active Site of TeNt Light Chain with Various Thiol-Containing Pseudopeptides Putatively Mimicking Synaptobrevin S'_1–S'_5 Binding Sites (A) and S_1–S_6 Binding Sites (B)

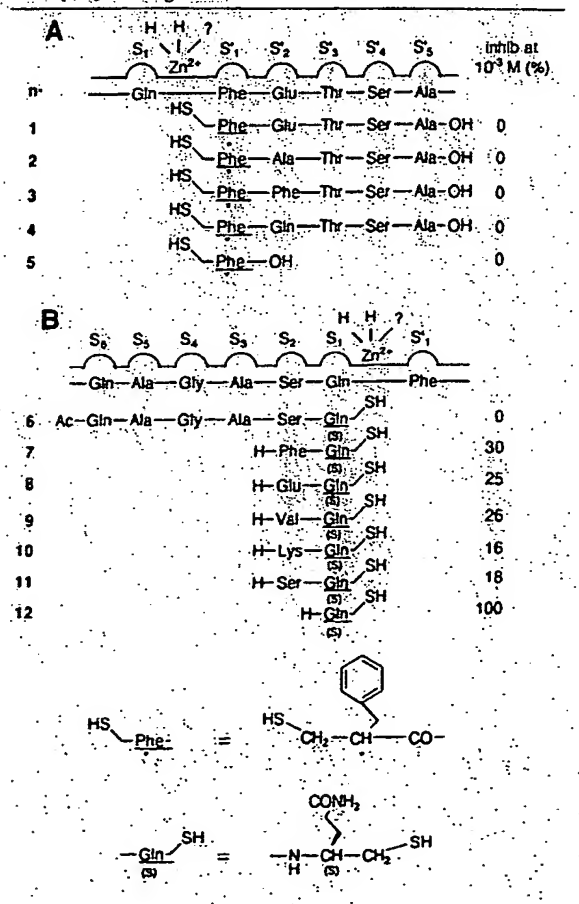
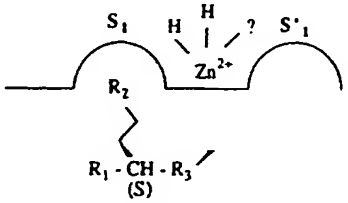


Table 2. Comparison of Inhibitory Potencies of Various Glutamine and Glutamate Analogues Containing, as a Zinc Ligand, a Thiol, Hydroxamate, Carboxylate, or Phosphonate Group: Importance of the Amine and the Chelation with Zinc



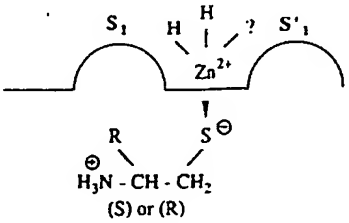
no.	R_1	R_2	R_3	inhib at 10^{-3} M (%)
	H_2N	$CONH_2$	COOH	0
19	H_2N	$CONH_2$	CH_2COOH	70
15	H_2N	$CONH_2$	CONHOH	0
18	H_2N	$CONH_2$	$CH_2CONHOH$	0
17	H_2N	$CONH_2$	CH_2CH_2SH	0
12	H_2N	$CONH_2$	CH_2SH	100
14	AcNH	$CONH_2$	CH_2SH	12
37	H_2N	COOH	CH_2SH	65
38	(Me)HN	COOH	CH_2SH	0
39	H_2N	COOH	PO_3H_2	0

On the basis of this result, various derivatives of glutamine bearing different functional groups capable of chelating the zinc atom of TeNt L-chain were tested. As reported in Table 2, the change of the position of the sulfanyl group in **17** strongly decreased its inhibitory effect as compared to **12**. The β- and γ-hydroxamate analogues **18** and **15** did not inhibit TeNt activity at 1 mM. Interestingly, a 70% inhibition was observed with 1 mM β-amino-homoglutamine **19**, while 1 mM L-glutamine was inactive. The phosphonate **39**[28] was a poor inhibitor. Thus, the sulfanyl group at the β-position represented the best zinc ligand to obtain significant inhibitory effect toward TeNt.

The need for a free primary amino group was investigated by introducing either an acetyl (**14**) or a methyl (**38**) group on the amino group of the β-amino-glutamine-thiol **12** or the β-amino-glutamate-thiol **37**, respectively. An almost complete abolition of any inhibitory effect was obtained with these modified compounds (Table 2).

Finally, various β-amino-thiols differing in their side chain structure were studied in order to explore the S_1 subsite specificity of TeNt (Tables 3 and 4). As shown in Table 3, the length of the aliphatic side chains does not seem to be very important, with only a slight preference for an ethylene linker (compare **12** to **40**). The charged or polar groups such as $-CO_2^-$, $-SO_3^-$, $-NH_3^+$, or $-CONH_2$ are more favorable than $-PO_3^{2-}$ for TeNt inhibition, and the chirality of the α-carbon is unimportant (compare **37** to **41** and **42** to **43**). The cyclic or aromatic side chains are poorly recognized (compound **48**) except when they are substituted at a meta position by a charged group (compound **53**). In Table 4, the K_i values of selected inhibitors are reported, and these indicate that a sulfonamide substituent is preferred in both linear ($K_i \sim 100$ μM) and aromatic ($K_i \sim 35$ μM) side chains.

Table 3. Exploration of the S_1 Subsite of TeNt Light Chain with β-Amino-thiols Having Various Aromatic and Aliphatic Side Chains



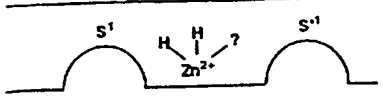
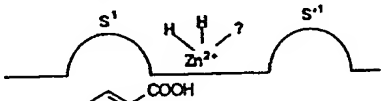
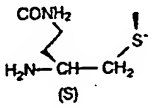
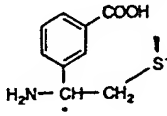
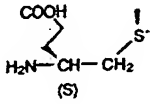
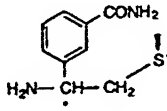
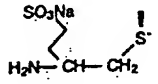
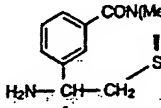
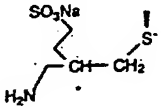
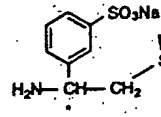
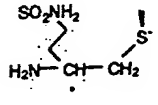
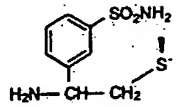
no.	config	side chain	inhib at 10^{-3} M (%)
12	(S)	R = -$(CH_2)_2$-$CONH_2$	100
40	(S)	R = -$(CH_2)_3$-$CONH_2$	75
37	(S)	R = -$(CH_2)_2$-COOH	65
41	(R)	R = -$(CH_2)_2$-COOH	73
42	(S)	R = -$(CH_2)_3$-COOH	92
43	(R)	R = -$(CH_2)_3$-COOH	69
44	(S)(R)	R = -$(CH_2)_4$-COOH	70
45	(S)	R = -$(CH_2)_4$-NH_3^+	90
46	(S)(R)	R = -$(CH_2)_2$-SONa	100
47	(S)(R)	R = -$(CH_2)_2$-PO_3H_2	46
48	(S)(R)	R = -CH_2-Ph	0
49	(S)(R)	R = -CH_2-(4-COOH)Ph	30
50	(S)(R)	R = -CH_2-(4-COOH)cHex	62
51	(S)(R)	R = -CH_2-(3-COOH)cHex	47
52	(S)(R)	R = -(4-COOH)Ph	0
53	(S)(R)	R = -(3-COOH)Ph	100

Discussion

The sequence of synaptobrevin at the C-terminus of the scissible bond (FETSA), putatively interacting with S'_1–S'_5 subsites of TeNt active site, was modified by substituting the amino-terminal group by a sulfanyl-methylene group, which is known to be a highly potent zinc ligand. For the five analogues described in Table 1A, no activity was detected even at a millimolar concentration of inhibitor. This is an unusual result for a zinc endopeptidase, as for most other enzymes of this group, the introduction of a zinc-coordinating group on molecules able to interact with at least one subsite of the catalytic domain has given lead compounds, which have been further optimized by structure–activity relationship analyses to give efficient inhibitors.[27,31,35–36] For instance, for angiotensin-converting enzyme (ACE) and for neprilysin (NEP), the sulfanyl analogues of Phe-Trp and Phe-Leu had inhibitory activities in the micromolar range.[37,38]

The peptide sequence of synaptobrevin at the N-terminus of the scissible bond (AGASQ), putatively interacting with the corresponding subsites of TeNt active site, was also modified by substituting the COOH terminal by a sulfanyl-methylene group (Table 1B). Weak activities were observed from pentapeptide to dipeptide, while the β-amino-glutamine-thiol **12** gave complete inhibition at 1 mM. Further experiments determined a K_i value of 250 μM for this compound (Table 4). Such types of inhibitor are well-known to inhibit efficiently aminopeptidase activities. Indeed, β-amino-glutamate-thiol, β-amino-methionine-thiol, and β-amino-lysine-thiol have K_i values ranging from 10^{-7} to 10^{-9} M for aminopeptidase A,[29] aminopeptidase N,[31] and aminopeptidase B,[30] respectively. Given the strict endopeptidase character of tetanus neurotoxin, this finding was quite surprising. Nevertheless, the requirement of a free primary amino group, common for aminopeptidase substrates or inhibitors, was further verified for TeNt by introducing an acetyl (**14**) or a

Table 4. Exploration of the TeNt Light Chain S_1 Subsite with Various β-Amino-thiols[a]

no.		K_I (μM)	no.		K_I (μM)
					
(12)		250 ± 35	(53)		250 ± 40
(37)		800 ± 50	(29a)		125 ± 25
(46)		300 ± 60	(29b)		250 ± 45
(54)		500 ± 45	(55)		40 ± 5
(24)		100 ± 5	(36)		35 ± 5

[a] The K_i values are the mean ± SEM of three independent experiments performed in duplicate.

methyl (38), which almost completely abolished inhibitory activity (Table 2). The requirement of its correct positioning was also verified with compound 54 by introducing a methylene between the primary amino group and the β-carbon of the thiol leading to a significant decrease in inhibitory efficiency (Table 4). This is unexpected for a strict endopeptidase. Two hypotheses could account for this behavior: (i) either an accessible glutamate (or aspartate) stabilizing this primary amino group by formation of hydrogen bonds is present in the active site of the enzyme, as demonstrated for APN;[40] or (ii) these inhibitors bind the zinc ion as bidentates involving both sulfanyl and amino groups.

The next step in this study was to optimize the nature and the positioning of the zinc chelating group. The sulfanyl group was found to be the most potent, as compared to other well-known zinc ligands such as hydroxamates, carboxylates, and phosphonates (Table 2). This was not unexpected because the sulfanyl group is one of the most efficient zinc ligands.

Finally, to improve the affinities of these β-amino-thiols for TeNt through efficient interactions with the putative S_1 subsite, various compounds previously synthesized and studied in our laboratory for aminopeptidase inhibition[26,27] and having different side chains have been tested (Tables 3 and 4). From these results, it can be concluded that the best interactions with the S_1 subsite are obtained with hydrophilic side chains bearing acid or amide moieties in the following decreasing order of efficacy SO_2NH_2 > SO_3H > $CONH_2$ > COOH. Furthermore, these functional groups have to be preferentially in γ positions of linear alkyl side chains, or even better in the meta position of a phenyl ring. The conformational restriction imposed by the phenyl ring, as compared to the linear alkyl chain, suggests a particularly well-fitted interaction in this case, between the acid or amide functional group and a putative hydrogen-bond acceptor group present in the S_1 subsite of TeNt. Moreover, inversion of the absolute configuration in 41 and 43 (compare respectively to 37 and 42) does not seem to be important in terms of inhibitory potency.

In addition, unlike most of the endopeptidases studied such as NEP, ACE, thermolysin, E.C. 24-15, and E.C. 24-16, TeNt does not recognize hydrophobic side chains at the S_1 subsite.

In conclusion, the present study is a preliminary step toward the characterization of the active site of this new type of zinc endopeptidase and the design of potent and selective inhibitors of TeNt. These data demonstrate the crucial role of the S_1 subsite in the active site cleft of TeNt, which seems to have some analogies with the S_1 subsite of acidic aminopeptidases such as aminopeptidase A, although it is well-known that TeNt does not have any aminopeptidase activity. The structure–activity relationship analysis performed on diversely substituted β-amino-thiols has established the critical position of a sulfanyl, a primary amino group, and a sulfonamide moiety for TeNt inhibition. This led to the design of β-amino-(4-sulfamoylphenyl)glycine-thiol 36, which with a K_i value of 35 μM is the first described relatively efficient in vitro inhibitor of TeNt proteolytic activity. Cell culture experiments are now in progress

in our laboratory in order to determine the membrane permeability of these β-amino-thiols. To be efficient in vivo, these inhibitors should be capable of entering the neuronal cytosol to inhibit the proteolytic activity of the toxin at its site of action.

Experimental Section

Chemistry. High-performance liquid chromatography (HPLC) grade solvents and solvents were from Solvants Documentation Synthese (Peypin en provence, France). Fmoc amino acids, solvents, and other reagents for solid-phase peptide synthesis (SPPS) were obtained from Perkin-Elmer. Chromatography was carried out with Merck silica gel Si 60 (40−63 μm). TLC was performed on silica gel (60 F 254, 0.2 mm thick; Merck). The final products were purified on an Applied Biosystem 151A HPLC apparatus with a C_{18} Vydac (ref 218TP510) and acetonitrile gradients performed with buffers A (H_2O, TFA 0.1% (v/v)) and B (CH_3CN/H_2O (7:3), TFA 0.09% (v/v)). The absorbance of eluted peaks was monitored at 214 nm. The purity of the final compounds was also checked by HPLC using a reverse-phase column (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) with the same elution buffers.

The structure of all the compounds was confirmed by ^1H NMR spectroscopy on a Bruker AC 270-MHz or Bruker AM 400-MHz spectrometer in DMSO-d_6 or $CDCl_3$ solutions (5×10^{-3} M) using HMDS as internal reference. The signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Satisfactory elemental analyses, performed at the university of P & M Curie Paris VI (Jussieu, Paris), were obtained (C, H, N) for all compounds. Mass spectral analyses for all the final compounds were achieved by Quad Service (Poissy, France) using the electrospray ionization technique (ESI). Optical rotations were measured on a Perkin-Elmer 241 polarimeter (1.0-dm cell) for MeOH solutions at 20 °C: $[\alpha]_D$ values are given in units of 10^{-1} deg $cm^2\ g^{-1}$. Melting points of the crystallized compounds were measured on an electrothermal apparatus and are reported uncorrected.

Abbreviation: Pya, pyrenylalanine.

General Procedure for Solid-Phase Peptide Synthesis. Protected peptide assemblies were carried out in NMP using either Wang/HMP resin or 2-chlorotrityl chloride resin on a ABI 431 peptide synthesizer (Perkin-Elmer) using the Fmoc/tBu strategy. Fmoc deprotection was achieved using 20% piperidine in NMP. Residues were coupled with 10-fold molar excess of Fmoc-amino acids using standard activation by DCC/HOBt in NMP. With HMP resin, the first amino acid could be coupled to the HMP resin by using DCC with DMAP catalysis, whereas the loading of 2-chlorotrityl chloride resin was performed by using DIEA in dry CH_2Cl_2. The amino acid side chain protecting groups used were 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg; Trt for Asn, Gln, and His; Boc for Lys; tBu for Asp, Glu, Ser, Thr, and Tyr. For HMP resin cleavage and peptide deprotection, the dry peptidyl resins were treated by TFA/H_2O/triisopropylsilane mixtures (92.5/5/2.5) for 2 h at room temperature.[41,42] For fully protected peptide cleavage from 2-chlorotrityl chloride resin, the dry peptidyl resins were stirred for 4 h with TFE/CH_2Cl_2 (2:8).[43]

Preparation of HSCH$_2$-CH(-CH$_2$Ph)-CO-NH-CH(R1)-CO-Thr-Ser-Ala-OH (Compounds 1−5). The various peptidyl-resins were prepared according to the solid-phase peptide synthesis procedure at a 50-μmol scale on HMP resin using (2*SR*)-3-(acetylsulfanyl)-2-benzylpropanoic acid in the last coupling step. This synthon was prepared as previously described.[44]

To deprotect the thiol group, the crude peptides were dissolved in degassed MeOH (2 mL/mmol) under inert atmosphere and 1 N NaOH (3 equiv) was added at 0 °C. The mixture was stirred for 3 h at room temperature. After acidification with HCl (2 N), the organic layers were evaporated, diluted in H_2O, and extracted with degassed EtOAc. The organic layers were washed with H_2O and brine, dried over Na_2SO_4, and evaporated to dryness to obtain the different compounds SH-free which were purified by HPLC.

R_1 = -CH$_2$-CH$_2$COOH (1): retention time = 14.6 and 15.4 min (A, B) (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) (26% yield); ^1H NMR (DMSO-d_6 + TFA) δ 1.00 (3H, d, $CH_3\gamma$(Thr)), 1.21 (3H, d, $CH_3\gamma$(Ala)), 1.74 (1H, m, CHβ(Glu)), 1.89 (1H, m, CHβ(Glu)), 2.20 (2H, t, $CH_2\gamma$(Glu)), 2.38−2.74 (4H, m, *SH*, CH_2S, *CH*CH_2S), 2.85 (2H, dd, CH_2Ph), 3.58 (2H, m, CHβ(Ser)), 3.97 (1H, m, CHβ(Thr)), 4.10−4.40 (4H, m, CHα(Ala), CHα(Thr), CHα(Ser), CHα(Glu)), 7.03−7.20 (5H, m, Ar), 7.60 (A) 7.70 (B) (1H, d, NH(Thr)), 7.82 (1H, m, NH(Ser)), 8.00 (1H, m, NH(Ala)), 8.16 (A) 8.23 (B) (1H, d, NH(Glu)).

R_1 = -CH$_3$ (2): retention time = 15.8 min (A, B) (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) (40% yield); ^1H NMR (DMSO-d_6 + TFA) δ 1.00 (3H, d, $CH_3\gamma$(Thr)), 1.20 (3H, d, $CH_3\gamma$(Ala)), 2.30−2.72 (4H, m, *SH*, CH_2S, *CH*CH_2S), 2.88 (2H, dd, CH_2Ph), 3.58 (2H, m, CHβ(Ser)), 3.97 (1H, m, CHβ(Thr)), 4.09−4.36 (4H, m, CHα(Ala), CHα(Thr), CHα(Ser), CHα(Ala)), 7.06−7.23 (5H, m, Ar), 7.60 (A) 7.70 (B) (1H, d, NH(Thr)), 7.78 (1H, m, NH(Ser)), 8.00 (1H, m, NH(Ala)), 8.11 (A) 8.22 (B) (1H, d, NH(Ala)).

R_1 = -CH$_2$Ph (3): retention time = 21.4 min (A, B) (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) (34% yield); ^1H NMR (DMSO-d_6 + TFA) δ 1.00 (3H, d, $CH_3\gamma$(Thr)), 1.20 (3H, d, $CH_3\gamma$(Ala)), 2.40−2.80 (4H, m, *SH*, CH_2S, *CH*CH_2S), 3.00 (2H, dd, CH_2Ph), 3.60 (2H, m, CHβ(Ser)), 4.00 (1H, m, CHβ(Thr)), 4.15 (1H, m, CHα(Ala)), 4.30 (2H, m, CHα(Thr), CHα(Ser)), 4.58 (1H, m, CHα(Phe)), 6.91−7.25 (5H, m, Ar), 7.80 (1H, d, NH(Thr)), 7.90 (1H, m, NH(Ser)), 8.03 (1H, m, NH(Ala)), 8.26 (1H, d, NH(Phe)).

R_1 = -CH$_2$-CH$_2$-CONH$_2$ (4): retention time = 13.4 and 14.3 min (A, B) (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) (38% yield); ^1H NMR (DMSO-d_6 + TFA) δ 1.00 (3H, d, $CH_3\gamma$(Thr)), 1.20 (3H, d, $CH_3\gamma$(Ala)), 1.75 (1H, m, CHβ(Gln)), 1.89 (1H, m, CHβ(Gln)), 2.10 (2H, t, $CH_2\gamma$(Gln)), 2.33−2.72 (4H, m, *SH*, CH_2S, *CH*CH_2S), 2.86 (2H, dd, CH_2Ph), 3.58 (2H, m, CHβ(Ser)), 3.97 (1H, m, CHβ(Thr)), 4.10−4.30 (4H, m, CHα(Ala), CHα(Thr), CHα(Ser), CHα(Gln)), 7.06−7.21 (5H, m, Ar), 7.67 (A) 7.81 (B) (1H, d, NH(Thr)), 7.82 (1H, m, NH(Ser)), 8.05 (1H, m, NH(Ala)), 8.22 (1H, d, NH(Glu)).

3,3′-(Disulfanediyl)bis[(2*SR*)-2-benzylpropanoic acid] (5): The 3,3′-(disulfanediyl)bis[(2*SR*)-2-benzylpropanoic acid] was prepared as previously described.[44]

Preparation of Symmetric Disulfides [Ac-Gln-Ala-Gly-Ala-Ser-*Gln*-(CH$_2$S-)]$_2$ and [H$_2$N-CH(R$_2$)-CONH-CH(CH$_2$-CH$_2$CONH$_2$)-CH$_2$S]$_2$ (6−11). The various protected commercially available BocHN-CH(R′2)-COOH as well as the protected peptide Ac-Gln(Trt)-Ala-Gly-Ala-Ser(tBu)OH, synthesized according to the general procedure of solid-phase peptide synthesis on 2-chlorotrityl chloride resin, were coupled to the symmetric disulfide [H$_2$N-CH(CH$_2$CH$_2$CONHTrt)-CH$_2$S]$_2$ (13) using BOP as coupling agent.[45] The protected peptides were treated by TFA/H_2O/triisopropylsilane mixtures (92.5/5/2.5), for 2 h at room temperature as previously described[41,42] and purified by HPLC.

[Ac-Gln-Ala-Gly-Ala-Ser-*Gln*-(CH$_2$S-)]$_2$ (6): retention time = 8.92 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−50% B in 15 min, flow rate 0.8 mL/min) (37% yield); ^1H NMR (DMSO-d_6) δ 1.17 (6H, d, 2 × $CH_3\beta$(Ala)), 1.50−1.65 (2H, m, $CH_2\beta$(Gln)), 1.68−1.84 (2H, m, $CH_2\beta$(Gln)), 1.78 (3H, s, CH_3-CO), 1.95−2.05 (4H, m, 2 × $CH_2\gamma$(Gln)), 2.74 (2H, m, CH_2S), 3.5−3.7 (4H, m, $CH_2\alpha$(Gly) and $CH_2\beta$(Ser)), 3.83 (1H, m, *CH*CH_2S), 4.1−4.25 (4H, m, CHα(Gln), CHα(Ala), CHα(Ala) and CHα(Ser)), 6.70 (2H, s, $CONH_2$), 7.16 (1H, s; $CONH_2$), 7.22 (1H, s, $CONH_2$), 7.63 (1H, d, NH), 7.87 (1H, d, NH), 7.95 (2H, t, NH), 8.06 (1H, d, NH), 8.13 (1H, t, NH).

R_2 = -CH$_2$Ph (7): retention time = 16.42 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0−80% B in 30 min, flow rate 0.8 mL/min) (41% yield); ^1H NMR (DMSO-d_6) δ 1.6−1.75 (2H, m, $CH_2\beta$(Gln)), 2.05 (2H, m, $CH_2\gamma$(Gln)), 2.6−2.75

(2H, dd, CH_2S), 2.9–3.05 (2H, dd, CH_2Ph), 3.9 (2H, m, CHα-(Phe) and CHα(Gln)), 6.75 and 7.15 (2H, s, $CONH_2$), 7.1–7.3 (5H, m, Ar), 8.1 (3H, s, NH_3^+), 8.4 (1H, d, –CONH–).

R_2 = -$(CH_2)_2$COOH (8): retention time = 6.64 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0–40% B in 15 min, flow rate 0.8 mL/min) (36% yield); ^{1}H NMR (DMSO-d_6) δ 1.6–1.75 (2H, m, $CH_2\beta$(Gln)), 2.1 (4H, m, $CH_2\gamma$(Gln) and $CH_2\beta$-(Glu)), 2.3 (2H, m, $CH_2\gamma$(Glu)), 2.8 (2H, dd, CH_2S), 3.95 (2H, m, CHα(Glu), CHα(Gln)), 7.95 (1H, d, –CONH–), 8.25 (3H, s, NH_3^+).

R_2 = -$CH(CH_3)_2$ (9): retention time = 14.18 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0–40% B in 15 min, flow rate 0.8 mL/min) (40% yield); ^{1}H NMR (DMSO-d_6) δ 0.85 (6H, m, $CH_3\delta$), 1.4–1.8 (5H, m, $CH_2\beta$(Gln), $CH_2\beta$(Leu), and $CH\gamma$(Leu)), 2.05 (2H, t, $CH_2\gamma$(Gln)), 2.7–2.9 (2H, dd, CH_2S), 3.6 (1H, m, CHα(Leu)), 3.95 (1H, m, CHα(Gln)), 6.7–7.15 (2H, s, $CONH_2$), 8.1 (3H, s, NH_3^+), 8.35 (1H, d, –CONH–).

R_2 = -$(CH_2)_4NH_2$ (10): retention time = 3.26 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0–30% B in 15 min, flow rate 0.8 mL/min) (29% yield); ^{1}H NMR (DMSO-d_6) δ 1.3–1.8 (8H, m, $CH_2\beta$(Gln), $CH_2\beta$(Lys), $CH_2\gamma$(Lys), $CH_2\delta$(Lys)), 2.05 (2H, t, $CH_2\gamma$(Gln)), 2.7 (2H, m, $CH_2\epsilon$(Lys)), 2.7–2.9 (2H, dd, CH_2S), 3.65, 3.95 (2H, m, CHα(Lys) and CHα(Gln)), 6.75–7.2 (2H, s, $CONH_2$), 7.7–8.1 (6H, s, NH_3^+ and NH_3^+(Lys)), 8.4 (1H, d, –CONH–).

R_2 = -CH_2OH (11): retention time = 5.15 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0–30% B in 15 min, flow rate 0.8 mL/min) (33% yield); ^{1}H NMR (DMSO-d_6) δ 1.6–1.75 (2H, m, $CH_2\beta$(Gln)), 2.05 (2H, t, $CH_2\gamma$(Gln)), 2.7–2.85 (2H, dd, CH_2S), 3.55 (1H, m, CHα(Ser)), 3.7 (2H, m, $CH_2\beta$(Ser)), 3.9 (1H, m, CHα(Gln)), 5.45 (1H, m, CH_2OH), 6.7–7.15 (2H, s, $CONH_2$), 8.05 (3H, s, NH_3^+), 8.3 (1H, d, –CONH–).

Preparation of 5,5′-(Disulfanediyl)bis[(4*S*)-4-amino-*N*-tritylpentanamide] (13) and 5,5′-(Disulfanediyl)bis[(4*S*)-4-aminopentanamide)] (12). The carboxylic acid function of (4*S*)-BocGln(Trt)-OH was transformed into alcohol by formation of the mixed anhydride with isobutyl chloroformate in DME at –15 °C followed by a reduction with sodium borohydride as previously described[24] yielding (4*S*)-4-(Boc-amino)-5-hydroxy-*N*-tritylpentanamide as a white solid (70% yield). Thioacetylation of this compound was then performed by a Mitsunobu reaction[25] to afford (4*S*)-5-(acetylsulfanyl)-4-(Boc-amino)-*N*-tritylpentanamide as a white solid (40% yield).

Deprotection of the acetyl group was achieved by using 1 N NaOH in methanol at room temperature for 3 h, and I_2 was then added until a persistent yellow color was obtained. The excess of iodine was reduced by $Na_2S_2O_3$, and the solution was evaporated in vacuo. The aqueous residue was acidified with 2 N HCl to pH 2–3 and extracted with EtOAc. After classical workup, 5,5′-(disulfanediyl)bis[(4*S*)-4-(*N*-Boc-amino)-*N*-tritylpentanamide] was obtained as a white solid (87% yield).

To a solution of 5,5′-(disulfanediyl)bis[(4*S*)-4-(*N*-Boc-amino)-*N*-tritylpentanamide] in acetic acid was added 6.5 equiv of boron trifluoride diethyl etherate.[46] After stirring for 1 h, the mixture was treated with a solution of NH_4OH (28%), the pH was adjusted to 9–10 with $KHCO_3$, and the aqueous layer was extracted with CH_2Cl_2. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated in vacuo to afford 5,5′-(disulfanediyl)bis[(4*S*)-4-amino-*N*-tritylpentanamide] (13) (82% yield): retention time = 24.3 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0–90% B in 30 min, flow rate 1.5 mL/min); ^{1}H NMR (DMSO-d_6) δ 1.8 (2H, m, $CH_2\beta$), 2.4 (2H, m, $CH_2\gamma$), 2.95 (2H, m, CH_2S), 3,15 (1H, m, CHα), 7.15 (15H, m, Trt), 8.30 (3H, s, NH_3^+), 8.65 (1H, s, CO*NH*Trt); $[\alpha]_D$ = +82.0 (c 0.87, MeOH); SM (ES) (M + H)$^+$ *m/z* = 780.3. Anal. ($C_{48}H_{50}N_4O_2S$).

5,5′-(Disulfanediyl)bis[(4*S*)-4-(*N*-Boc-amino)-*N*-tritylpentanamide] was treated by a TFA/H_2O/triisopropylsilane mixture (92.5/5/2.5) for 2 h at room temperature as previously described.[41,42] **5,5′-(Disulfanediyl)bis[(4*S*)-4-aminopentanamide] (12)** was thus obtained as a yellow oil by precipitation in diethyl ether/hexane (1/1) (98% yield): retention time = 9.97 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0–40% B in 15 min, flow rate 0.8 mL/min); ^{1}H NMR (DMSO-d_6) δ 1.8 (2H, m, $CH_2\beta$), 2.2 (2H, m, $CH_2\gamma$), 2.9 (2H, m, CH_2S), 3.3 (1H, m, CHα), 6.9–7.4 (2H, s, $CONH_2$), 8.1 (3H, s, NH_3^+); SM (ES) (M + H)$^+$ *m/z* = 295.2. Anal. ($C_{10}H_{22}N_4O_2S_2$).

5,5′-(Disulfanediyl)bis[(4*S*)-4-(acetylamino)pentanamide] (14). [H_2N-CH($CH_2CH_2CONHTrt$)-CH_2S]$_2$ (13) was coupled to anhydride acetic acid in DMF with 2 equiv of DIEA. The *N*-tritylamide was deprotected as previously described[41,42] and purified by semipreparative HPLC on a C18 Vydac column (29% yield): retention time = 10.37 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0–40% B in 15 min, flow rate 0.8 mL/min); ^{1}H NMR (DMSO-d_6) δ 1.5–1.7 (2H, m, $CH_2\beta$), 1.7 (3H, s, CH_3CONH), 2.0 (2H, t, $CH_2\gamma$), 2.75 (2H, m, CH_2S), 3.85 (1H, m, CHα), 6.66 (1H, s, $CONH_2$), 7.2 (1H, s, $CONH_2$), 7.7 (1H, d, CH_3CONH).

(2*S*)-2-Amino-*N*^{1}-hydroxypentanediamide (15). BocGln-(Trt)OH was coupled with benzyloxyamine hydrochloride (1 molar equiv) using BOP as coupling agent[45] to afford (2*S*)-*N*^{1}-benzyloxy-2-(Boc-amino)-*N*^{5}-tritylpentanediamide: $[\alpha]_D$ = –17.8 (c 0.95, MeOH) (91% yield).

The benzyl protecting group was cleaved by hydrogenolysis with 10% Pd/C as catalyst to yield (2*S*)-2-(Boc-amino)-*N*^{1}-hydroxy-*N*^{5}-tritylpentanediamide, and the trityl group was cleaved as previously described[41,42] to afford (2*S*)-2-amino-*N*^{1}-hydroxypentanediamide (15) (36% yield): R_f = 0.23 in propanol-2/NH_4OH/H_2O (7/1/1); ^{1}H NMR (DMSO-d_6) δ 1.55 (2H, m, $CH_2CH_2CONH_2$), 2.01 (2H, t, CH_2CONH_2), 2.56 (1H, m, H_3^+NCH), 7.00 (1H, s, $CONH_2$), 7.34 (1H, s, $CONH_2$), 7.73 (3H, br s, NH_3^+), 8.90 (1H, s, OH), 10.64 (1H, s, NH); SM (ES) (M + H)$^+$ *m/z* = 162.0. Anal. ($C_5H_{11}N_3O_3$).

(3*S*)-3-(Boc-amino)-6-oxo-(tritylamino)hexanoic Acid (16). To 0.5 M (*S*)-BocGln(Trt)-OH in THF was added *N*-methylmorpholine (1.1 molar equiv), followed by isobutyl chloroformate (1.1 molar equiv) at –15 °C. After 20 min of stirring at this temperature, the white precipitate was filtered off and washed with THF. To this solution was added an ethereal solution of diazomethane (2.0 molar equiv), and the yellow reaction mixture stirred at room temperature during 1 h. After evaporation of the excess diazomethane and removal of the solvent under reduced pressure, the diazoketone was taken up in EtOAc, washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude diazoketone, dissolved in dioxane was gradually added into a stirring mixture of Ag_2O (0.18 equiv), anhydrous Na_2CO_3 (0.96 equiv), $Na_2S_2O_3$·$5H_2O$ (0.23 equiv), H_2O heated at 50 °C. After 1 h at reflux, the reaction mixture was cooled, diluted with water, filtered, and extracted with three portions of diethyl ether. The aqueous phase was acidified with $KHSO_4$ (1 N), extracted with ethyl acetate, washed with brine, dried with Na_2SO_4, and evaporated in vacuo (75% yield): R_f = 0.57 in AcEt/CH_2Cl_2 (1/3); ^{1}H NMR (DMSO-d_6) δ 1.33 (9H, s, tBu), 1.39–1.58 (2H, m, $CH_2CH_2CONHTrt$), 2.16–2.27 (4H, m, CH_2-$CONHTrt$ and CH_2COOH), 3.69 (1H, m, BocNH*CH*), 6.61 (1H, d, Boc*NH*), 7.06–7.25 (15H, m, Trt), 8.50 (1H, s, *NH*Trt), 12.1 (1H, br s, COOH); SM (ES) (M + H)$^+$ *m/z* = 503.7. Anal. ($C_{30}H_{34}N_2O_5$) C, H, N.

6,6′-(Disulfanediyl)bis[(4*S*)-4-aminohexanamide] (17). This compound was synthesized from 16 using the procedure described for compound 12 (94% yield): ^{1}H NMR (DMSO-d_6 + TFA) δ 1.90 (2H, m, $CH_2CH_2CONH_2$), 2.30 (2H, t, CH_2-$CONH_2$), 2.90 (1H, ABX *J* = 16, 6 Hz, CH_2S), 3.08 (1H, ABX *J* = 16, 6 Hz, CH_2S), 3.44 (1H, m, BocNH*CH*), 8.03 (3H, br s, NH_3^+); SM (ES) (M + H)$^+$ *m/z* = 323.7. Anal. ($C_{12}H_{26}N_4O_2S_2$).

(3*S*)-3-Amino-*N*^{1}-hydroxyhexanediamide (18). This compound was synthesized from 16 using the procedure described for compound 15 (33% yield): ^{1}H NMR (DMSO-d_6) δ 1.68 (2H, m, $CH_2CH_2CONH_2$), 2.26 (2H, t, CH_2CONH_2), 2.40 (2H, t, CH_2-$CONHOH$), 3.30 (1H, m, H_3^+NCH), 7.12 (1H, s, $CONH_2$), 7.60 (1H, s, $CONH_2$), 7.84 (3H, br s, NH_3^+), 8.92 (1H, s, OH), 10.66 (1H, s, NH); SM (ES) (M + H)$^+$ *m/z* = 176.0. Anal. ($C_6H_{13}N_3O_3$).

(3*S*)-3-Amino-6-oxo-aminohexanoic Acid (19). This compound was obtained by deprotection of the tritylamide of compound 16 as previously described[41,42] (92% yield): R_f = 0.34 in propanol-2/NH_4OH/H_2O (7/1/1); ^{1}H NMR (DMSO-d_6) δ 1.73

(2H, m, $CH_2CH_2CONH_2$), 2.15 (2H, t; CH_2CONH_2), 2.57 (2H, ABX J = 16, 6 Hz, CH_2COOH), 3.35 (1H, m, BocNHCH), 6.86 (1H, s, CONH_2), 7.33 (1H, s, CONH_2), 7.86 (3H, br s, NH_3^+); SM (ES) $(M + H)^+$ m/z = 161.3. Anal. ($C_6H_{12}N_2O_3$).

4,4'-(Disulfanediyl)bis[methyl (2*SR*)-2-(Cbz-amino)butanoate] or DL-(*Z*)-Homocystine-OMe (20). This compound was synthesized as previously described[32] (96% yield): R_f = 0.46 in AcEt/CH_2Cl_2 (1/9); ^{1}H NMR (DMSO-d_6) δ 1.83–2.09 (2H, m, CH_2CH_2S), 2.68 (2H, t, CH_2S), 3.58 (3H, s, COOCH_3), 4.13 (1H, m, CHCOOMe), 4.98 (2H, s, CH_2 Ph), 7.28 (5H, m, Ph), 7.75 (1H, d, BzlOCONH). Anal. ($C_{26}H_{32}N_2O_8S_2$) C, H, N.

Methyl (2*SR*)-4-(*tert*-Butylsulfamoyl)-2-(Cbz-amino)-butanoate (21). To a cold (0 °C) solution of (*Z*)-homocystine-OMe (20) in MeOH (0.7 mL/mmol) and CCl_4 (3.5 mL/mmol) was bubbled Cl_2 (gas) for 1 h[32] in order to obtain methyl (2*SR*)-2-(Cbz-amino)-4-(chlorosulfonyl)butanoate. To a solution of the latter in CH_2Cl_2 (1.8 mL/mmol) was added *tert*-butylamine. After evaporation and classical workup, the residue was purified on a silica gel column, using cHex/CH_2Cl_2/EtOAc (5/3/2) as eluent (77% yield): R_f = 0.42 in AcEt/CH_2Cl_2 (1/9); ^{1}H NMR (DMSO-d_6) δ 1.18 (9H, s, tBu), 1.88–2.13 (2H, m, CH_2-CH_2SO_2NH), 2.85–3.10 (2H, m, CH_2SO_2NH), 3.58 (3H, s, COOCH_3), 4.21 (1H, m, CHCOOMe), 4.99 (2H, s, CH_2Ph), 6.86 (1H, s, NHtBu), 7.29 (5H, m, Ph), 7.85 (1H, d, BzlOCONH). Anal. ($C_{17}H_{26}N_2O_6S$) C, H, N.

(3*SR*)-*N*-*tert*-Butyl-3-(Cbz-amino)-4-hydroxybutanesulfonamide (22). The N-protected amino ester 21 (1 equiv) was dissolved in EtOH/H_2O (1/1). $NaBH_4$ (4 equiv) was added, and the mixture was heated at 50 °C for 6 h. The reaction was stopped with 1 N HCl, and the mixture was extracted with EtOAc. After classical workup, (3*SR*)-*N*-*tert*-butyl-3-(Cbz-amino)-4-hydroxybutanesulfonamide was obtained (74% yield): R_f = 0.18 in MeOH/CH_2Cl_2 (4/96); ^{1}H NMR (DMSO-d_6) δ 1.20 (9H, s, tBu), 1.64–1.99 (2H, m, $CH_2CH_2SO_2NH$), 2.90 (2H, t, CH_2SO_2NH), 3.30 (2H, m, CH_2OH), 3.50 (1H, m, $CH$$CH_2OH$), 4.72 (1H, t, CH_2OH), 4.97 (2H, d, CH_2Ph), 6.76 (1H, s, NHtBu), 7.11 (1H, d, BzlOCONH), 7.29 (5H, m, Ph). Anal. ($C_{16}H_{26}N_2O_5S$) C, H, N.

(3*SR*)-*N*-*tert*-Butyl-3-(Cbz-amino)-4-(acetylsulfanyl)butanesulfonamide (23). The thioacetylation of (3*SR*)-*N*-*tert*-butyl-3-(Cbz-amino)-4-hydroxybutanesulfonamide was then performed by a Mitsunobu reaction[25] to afford (3*SR*)-*N*-*tert*-butyl-3-(Cbz-amino)-4-(acetylsulfanyl)butanesulfonamide as a white solid (60% yield): R_f = 0.24 in Et_2O/cyclohexane (75/25); ^{1}H NMR (DMSO-d_6) δ 1.17 (9H, s, tBu), 1.70–1.90 (2H, m, $CH_2CH_2SO_2NH$), 2.28 (3H, s, SCOCH_3), 2.82 (1H, ABX J = 13.6 Hz, $CHCH_2SCOCH_3$), 2.89 (2H, t, CH_2SO_2NH), 3.03 (1H, ABX J = 13.6 Hz, $CHCH_2SCOCH_3$), 3.60 (1H, m, $CHCH_2$-$SCOCH_3$), 4.98 (2H, s, CH_2Ph), 6.80 (1H, s, SO_2NH), 7.29 (5H, m, Ph), 7.37 (1H, d, BzlOCONH). Anal. ($C_{18}H_{28}N_2O_5S_2$) C, H, N.

(3*SR*)-3-Amino-4-sulfanylbutanesulfonamide (24). The deprotection of the sulfonyl group was performed as described for compounds 1–5. Then, the cleavage of the trityl group[41,42] was followed by HF treatment.[47] (3*SR*)-3-Amino-4-sulfanylbutanesulfonamide (24) was thus obtained as a yellow oil (98% yield): ^{1}H NMR (DMSO-d_6) δ 2.00 (2H, m, $CH_2CH_2SO_2NH_2$), 2.73 (2H, m, CH_2SH), 3.07 (2H, m, $CH_2SO_2NH_2$), 3.30 (1H, m, $CHCH_2SH$), 6.85 (3H, brs, NH_3^+); SM (ES) $(M + H)^+$ m/z = 185.4. Anal. ($C_4H_{12}N_2O_2S_2$).

DL-Diethyl (Boc-amino)-(3-carboxyphenyl)glycinate (25). DL-(3-Carboxyphenyl)glycine, prepared as previously described,[27] was refluxed in EtOH with 5 equiv of $SOCl_2$ (100%). The resulting compound was N-protected by a *tert*-butyloxycarbonyl group with a procedure previously described[48] to obtain DL-diethyl (Boc-amino)(3-carboxyphenyl)-glycinate (25) using Boc_2O, NEt_3 in DMF (100% yield): R_f = 0.61 in EtOAc/heptane (1/1); ^{1}H NMR (DMSO-d_6) δ 1.07 (3H, t, $CHCOOCH_2CH_3$), 1.26 (3H, t, $C_6H_4COOCH_2CH_3$), 1.33 (9H, s, tBu), 4.04 (2H, q, $CHCOOCH_2CH_3$), 4.27 (2H, q, C_6H_4-$COOCH_2CH_3$), 5.22 (1H, d, $CHCOOCH_2CH_3$), 7.46 (1H, t, Ar), 7.60 (1H, d, Ar), 7.85 (2H, d, Ar and BocNH), 7.93 (1H, s, Ar). Anal. ($C_{18}H_{25}NO_6$) C, H, N.

3-[(2*SR*)-2-(Boc-amino)-1-hydroxyethyl]benzoic Acid (26). The α-amino ester 25 (1 equiv) was reduced in dry EtOH/THF (8 mL/mmol) at 0 °C by $NaBH_4$ (4 equiv) and LiCl (4 equiv) as previously described.[49] This compound (1 equiv) was dissolved in MeOH (2 mL/mmol), and 1 N NaOH (3 equiv) was added at 0 °C. After classical treatment, 3-[(2*SR*)-2-(Boc-amino)-1-hydroxyethyl]benzoic acid (26) (83% yield) was obtained: R_f = 0.58 in CH_2Cl_2/MeOH/AcOH (9/1/0.5); ^{1}H NMR (DMSO-d_6) δ 1.30 (9H, s, tBu), 3.45 (2H, m, CH_2OH), 4.51 (1H, q, $CHCH_2OH$), 4.27 (1H, br s, CH_2OH), 7.30 (1H, d, BocNH), 7.37 (1H, t, Ar), 7.47 (1H, d, Ar), 7.75 (1H, d, Ar), 7.83 (1H, s, Ar). Anal. ($C_{14}H_{19}NO_5$) C, H, N.

Preparation of 3-[(2*SR*)-2-(Boc-amino)-1-hydroxyethyl]benzamide (27a) and 3-[(2*SR*)-2-(Boc-amino)-1-hydroxyethyl]-*N*,*N*-dimethylbenzamide (27b). To a cold (−15 °C) solution of 26 (1 equiv) in DMF were successively added *N*-methylmorpholine (1.1 equiv) and ^{t}BuOCOCl (1.1 equiv). After 15 min, the precipitated *N*-methylmorpholine hydrochloride was removed by filtration. For preparation of 27a, a solution of NH_3 (2 M) in DME (60 equiv) was added at −15 °C. For preparation of 27b, a solution of dimethylamine (3 equiv) and triethylamine (3 equiv) in DMF was added at −15 °C. In both cases the mixture was stirred at room temperature for 4 h. After evaporation in vacuo, the residue was washed with H_2O and brine, dried over Na_2SO_4, and evaporated in vacuo. The product 27a was purified by flash chromotography on a silica gel column, using CH_2Cl_2/MeOH/AcOH (9/1/0.5) as eluent (46% yield): R_f = 0.37 in CH_2Cl_2/MeOH/AcOH (9/1/0.5); ^{1}H NMR (DMSO-d_6) δ 1.31 (9H, s, tBu), 3.45 (2H, t, CH_2OH), 4.50 (1H, q, $CHCH_2OH$), 4.77 (1H, t, CH_2OH), 7.20 (1H, d, BOCNH), 7.28 (1H, s, CONH_2), 7.32 (1H, d, Ar), 7.36 (1H, t, Ar), 7.67 (1H, d, Ar), 7.76 (1H, s, Ar), 7.87 (1H, s, CONH_2). Anal. ($C_{14}H_{20}N_2O_4$) C, H, N.

The product 27b was purified by flash chromatography on a silica gel column, using CH_2Cl_2/MeOH/AcOH (9/1/0.5) as eluent (68% yield): R_f = 0.60 in CH_2Cl_2/EtOAc/AcOH (9/1/0.5); ^{1}H NMR (DMSO-d_6) δ 1.32 (9H, s, tBu), 2.85 (3H, s, CON-$(CH_3)CH_3$), 2.93 (3H, s, CON$(CH_3)CH_3$), 3.45 (2H, d, CH_2OH), 4.50 (1H, m, $CHCH_2OH$), 7.17–7.34 (5H, m, Ar and BocNH). Anal. ($C_{16}H_{24}N_2O_4$) C, H, N.

Preparation of 3-[(2*SR*)-1-(Acetylsulfanyl)-2-(Boc-amino)ethyl]benzamide (28a) and 3-[(2*SR*)-1-(Acetylsulfanyl)-2-(Boc-amino)ethyl]-*N*,*N*-dimethylbenzamide (28b). The free hydroxy compound 27a (respectively 27b) (1 equiv) was dissolved in DMF (0.2 mmol/mL), then TEA (2.7 molar equiv) and methanesulfonyl chloride were added at −10 °C.[50] The reaction mixture was stirred at room temperature for 3 h. The DMF was evaporated, and the residue was taken up in EtOAc and treated by classical workup. To a solution of the mesylate (1 equiv) in DMF (5 mL/mmol) was added at 0 °C 3 equiv of potassium thioacetate. After stirring overnight at room temperature and evaporation to dryness, the residue was taken up in EtOAc, washed with water and brine, dried, and evaporated in vacuo.

Compound 28a: R_f = 0.21 in *n*-heptane/EtOAc/AcOH (5/5/0.5) (71% yield); ^{1}H NMR (DMSO-d_6) δ 1.29 (9H, s, tBu), 2.27 (3H, s, SCOCH_3), 2.97 (1H, dd, CH_2SCOCH_3), 3.15 (1H, dd, CH_2SCOCH_3), 4.54 (1H, q, $CHCH_2SCOCH_3$), 7.33 (1H, s, CONH_2), 7.36 (1H, t, Ar), 7.42 (1H, d, Ar), 7.53 (1H, d, BocNH), 7.70 (1H, d, Ar), 7.80 (1H, s, Ar), 7.93 (1H, s, CONH_2). Anal. ($C_{16}H_{22}N_2O_4S$) C, H, N.

Compound 28b: R_f = 0.21 in *n*-heptane/EtOAc/AcOH (5/5/0.5) (38% yield); ^{1}H NMR (DMSO-d_6) δ 1.30 (9H, s, tBu), 2.28 (3H, s, SCOCH_3), 2.83 (3H, s, CON$(CH_3)CH_3$), 2.90 (3H, s, CON$(CH_3)CH_3$), 3.00 (1H, dd, CH_2SCOCH_3), 3.15 (1H, dd, CH_2-$SCOCH_3$), 4.54 (1H, m, $CHCH_2SCOCH_3$), 7.19–7.38 (4H, m, Ar), 7.53 (1H, d, BocNH). Anal. ($C_{18}H_{28}N_2O_4S$) C, H, N.

Preparation of 3,3'-[(Disulfanediyl)bis((2*SR*)-2-aminoethylene)]bis(benzamide) (29a) and 3,3'-[(Disulfanediyl)-bis((2*SR*)-2-aminoethylene)]bis(*N*,*N*-dimethylbenzamide) (29b). Deprotection of the acetyl and Boc groups of compounds 28a,b was obtained as described for compound 12.

Compound 29a: R_f = 0.14 in CH_2Cl_2/MeOH/AcOH (5/5/0.5) (82% yield); ^{1}H NMR (DMSO-d_6) δ 3.30 (2H, m, CH_2S), 4.50

(1H, m, *CH*CH_2S), 7.43 (1H, s, CO*NH*$_2$), 7.46 (1H, t, Ar), 7.56 (1H, d, Ar), 7.85 (1H, d, Ar), 7.99 (2H, s, CO*NH*$_2$ and Ar), 8.58 (3H, br s, *NH*$_3^+$); SM (ES) (M + H)$^+$ *m/z* = 391.6. Anal. ($C_{18}H_{22}N_4O_2S_2$).

Compound 29b: R_f = 0.33 in 2-propanol/NH_4OH (9/0.5) (52% yield); ^{1}H NMR (DMSO-d_6) δ 2.82 (3H, s, CON(CH_3)-*CH*$_3$), 2.92 (3H, s, CON(*CH*$_3$)CH$_3$), 3.23 (2H, m, *CH*$_2$S), 4.49 (1H, m, *CH*CH_2S), 7.33–7.50 (4H, m, Ar), 8.50 (3H, br s, NH_3^+); SM (ES) (M + H)$^+$ *m/z* = 447.5. Anal. ($C_{22}H_{30}N_4O_2S_2$).

Preparation of 3-(*N*-Benzylsulfamoyl)benzoic Acid (30). To a cold (0 °C) solution of 3-(chlorosulfonyl)benzoic acid (Aldrich) in CH_2Cl_2 was added benzylamine (3.5 equiv). The mixture was stirred for 30 min. After evaporation in vacuo and acidification with $KHSO_4$ (1 N), the aqueous residue was extracted three times with EtOAc (95% yield): R_f = 0.38 in toluene/AcOH (17/3); ^{1}H NMR (DMSO-d_6) δ 3.97 (2H, d, *CH*$_2$ Ph), 7.13–7.24 (5H, m, Ph), 7.63 (1H, t, Ar), 7.94 (1H, d, Ar), 8.08 (1H, d, Ar), 8.26 (1H, s, Ar), 8.29 (1H, t, SO_2*NH*), 13.4 (1H, br s, COOH). Anal. ($C_{14}H_{13}NO_4S$) C, H, N.

***N*-Benzyl-3-formylbenzenesulfonamide (31).** The carboxylic acid function of 3-(*N*-benzylsulfamoyl)benzoic acid (30) was transformed into alcohol to obtain *N*-benzyl-3-(hydroxymethyl)benzenesulfonamide by formation of the mixed anhydride with isobutyl chloroformate in DME at −15 °C followed by a reduction with sodium borohydride as previously described[24] (94% yield). Swern oxidation was performed with a cold (−78 °C) solution of oxalyl chloride (1.2 equiv) and dimethyl sulfoxide (3 equiv) diluted with dichloromethane.[51] After 5 min at −78 °C was added a solution of alcohol (1 equiv) in CH_2Cl_2 with dimethyl sulfoxide (3 equiv); stirring was continued for an additional 15 min. Triethylamine (5 equiv) was added, and the mixture was stirred for 5 min and then allowed to reach room temperature. After classical workup, *N*-benzyl-3-formylbenzenesulfonamide (96% yield) was obtained (31): R_f = 0.43 in toluene/AcOH (17/3); ^{1}H NMR (DMSO-d_6) δ 4.00 (2H, d, CH_2 Ph), 7.18 (5H, br s, Ph), 7.74 (1H, t, Ar), 8.02 (1H, d, Ar), 8.08 (1H, d, Ar), 8.20 (1H, s, Ar), 8.35 (1H, t, SO_2*NH*), 10.03 (1H, s, *CHO*). Anal. ($C_{14}H_{13}NO_3S$) C, H, N.

***N*-Benzyl-3-[(*SR*)-[(diphenylmethyl)amino]-cyanomethyl]benzenesulfonamide (32).** To a solution of aldehyde 31 (1 equiv) and KCN (1.01 equiv) in freshly distillated MeOH were added aminodiphenylmethane (1.15 equiv) and acetic acid (2.3 equiv). The mixture was stirred at reflux overnight. After evaporation in vacuo, the residue was purified by flash chromatography on a silica gel column, using EtOAc/cyclohexane (22/78) as eluent (63% yield): R_f = 0.33 in EtOAc/cyclohexane (1/2); ^{1}H NMR (DMSO-d_6) δ 3.97 (2H, d, *CH*$_2$Ph), 4.28 (1H, ABX J = 12.3 Hz, Ph(Ph)C*HNH*), 4.70 (1H, d J = 12 Hz, *CHCN*), 5.03 (1H, d J = 3 Hz, Ph(Ph)*CH*NH), 7.12–7.50 (15, m, 3 × Ph), 7.58 (1H, t, Ar), 7.72–7.74 (2H, m, Ar), 7.93 (1H, s, Ar), 8.24 (1H, t, *NH*Bzl). Anal. ($C_{28}H_{25}N_3O_2S$) C, H, N.

***N*-Benzyl-3-[(*SR*)-[(diphenylmethyl)amino]-(methoxycarbonyl)methyl]benzenesulfonamide (33).** The nitrile group of 32 was transformed into a carboxamide group by HCl(g) in anhydrous MeOH (75%) in order to obtain *N*-benzyl-3-[(2*SR*)-2-[(diphenylmethyl)amino]ethanamido]benzenesulfonamide. The carboxamide was combined with a 15-fold excess (by weight) of Amberlite IR-120 acidic resin in methanol.[52] The mixture was gently stirred and warmed (60 °C) for 2 days. The product was recovered by collecting the resin in a column and eluting slowly with a mixture of MeOH/NEt_3 (2/1) as eluent (67% yield): R_f = 0.52 in CH_2Cl_2/MeOH/AcOH (9/0.2/0.2); ^{1}H NMR (DMSO-d_6) δ 3.42 (1H, ABX J = 10.15 Hz, Ph-(Ph)C*HNH*), 3.58 (3H, s, $COOCH_3$), 3.94 (2H, d, *CH*$_2$Ph), 4.23 (1H, d J = 10 Hz, *CH*CN), 4.70 (1H, d J = 5 Hz, Ph(Ph)*CH*NH), 7.13–7.37 (15, m, 3 × Ph), 7.49 (1H, t, Ar), 7.52 (1H, d, Ar), 7.68 (1H, d, Ar), 7.76 (1H, s, Ar), 8.16 (1H, t, *NH*Bzl). Anal. ($C_{29}H_{28}N_2O_4S$) C, H, N; C: calcd, 69.58; found, 70.01.

***N*-Benzyl-3-[(2*SR*)-2-[(diphenylmethyl)amino]-1-hydroxyethyl]benzenesulfonamide (34).** Reduction of ester of 33 was performed using the procedure described for compound 26.[49] The product was purified by flash chromatography on a silica gel column, using EtOAc/cyclohexane (15/85) as eluent (96% yield): R_f = 0.31 in EtOAc/cyclohexane (3/7); ^{1}H NMR (DMSO-d_6 + TFA) δ 3.82 (2H, t, *CH*$_2$OH), 3.96 (1H, t, *CH*CH_2-OH), 4.05 (1H, t, CH_2*OH*), 3.98 (2H, d, *CH*$_2$Ph), 5.35 (1H, s, Ph(Ph)*CH*N), 7.03–7.62 (15H, m, 3 × Ph) and (2H, m, Ar), 7.80 (1H, d, Ar), 7.87 (1H, s, Ar), 8.17 (1H, t, *NH*Bzl). Anal. ($C_{28}H_{28}N_2O_3S$) C, H, N.

Preparation of *N*-Benzyl-3-[(2*SR*)-1-(acetylsulfanyl)-2-[(diphenylmethyl)amino]ethyl]benzenesulfonamide (35). This compound was prepared from 34 according to the procedure described for 23. The product was purified on a silica gel column using Et_2O/cyclohexane (1/9) as eluent (43% yield): R_f = 0.36 in EtOAc/*n*-heptane (3/7); ^{1}H NMR (DMSO-d_6 + TFA) δ 2.10 (3H, s, $SCOCH_3$), 3.39 (1H, t, *CH*CH_2-$SCOCH_3$), 3.80 (1H, ABX J = 12.4 Hz, $CHCH_2SCOCH_3$), 4.16 (1H, ABX J = 12.4 Hz, $CHCH_2SCOCH_3$), 4.00 (2H, s, *CH*$_2$Ph), 5.31 (1H, s, Ph(Ph)*CH*N), 7.05–7.54 (20H, m, 3 × Ph) and (2H, m, Ar), 7.82 (1H, d, Ar), 7.92 (1H, s, Ar), 8.16 (1H, t, *NH*Bzl). Anal. ($C_{30}H_{30}N_2O_3S_2$) C, H, N.

3-[(2*SR*)-2-Amino-1-sulfanylethyl]benzenesulfonamide (36). This compound was prepared from 35 according to the procedure described for 24. The single modification was that HF cleavage was run at room temperature: retention time = 18.4 min (C_{18} Nucleosil column, 0–80% B in 30 min; flow 1.2 mL/min); ^{1}H NMR (DMSO-d_6) δ 2.54 (1H, t, CH_2*SH*), 2.90–3.04 (2H, m, *CH*$_2$SH), 4.42 (1H, m, *CH*CH_2SH), 7.42 (2H, s, SO_2*NH*$_2$), 7.62 (2H, s, Ar), 7.80 (1H, s, Ar), 7.90 (1H, s, Ar), 8.50 (3H, br s, *NH*$_3^+$); SM (ES) (M + H)$^+$ *m/z* = 233.0. Anal. ($C_8H_{12}N_2O_2S_2$).

Compounds 37,[26] 38,[27] 39,[25] 40,[26] 41,[27] 42,[25] 43,[26] 44,[26] 45,[30] 46,[26] 47,[26] 48,[31] 49,[27] 50,[27] 51,[27] 52,[27] 53,[27] 54,[53] and 55[53] were prepared as previously described.

Inhibitory Potency. Enzymatic studies were performed using the fluorescent synaptobrevin derivative [Pya^{88}]S 39-88 as a substrate according to the protocol described by Solheihac et al.[34] with slight modifications: 250 ng of TeNt-L chain (Pasteur-Meyrieux, France), purified according to the procedure of Solheihac et al.,[34] was preincubated for 30 min at 37 °C with increasing concentrations of inhibitor in 90 μL of 20 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol. A 10-μL solution of 100 μM [Pya^{88}]S 39-88 in buffer was then added (10 μM final concentration), and the mixture was kept for 60 min at 37 °C in the dark. The reaction was stopped by adding 50 μL of 0.2 M HCl.

The fluorescent cleavage product was separated from the fluorescent substrate by reverse-phase HPLC, on a Nucleosil C_8 column (300 Å, 7 μm, 70 × 4 mm) with a LC-10AS Shimadzu apparatus and quantified by a RF-35 Shimadzu fluorimeter detector (on line) (excitation 343 nm, emission 377 nm).

The elution buffers used were A (H_2O, TFA 0.05% (v/v)) and B (CH_3CN/H_2O (9:1, v/v), TFA 0.038% (v/v)). The metabolites were eluted with a gradient of 28–50% in B for 2.5 min at a flow rate of 1 mL/min, followed by a gradient of 50–100% in B for 0.5 min, and isocratic at 100% of B for 2 min. In these conditions, the retention times of the fluorescent cleavage product and the fluorescent substrate peaks were respectively 3.4 and 5.7 min.

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Differential Inhibition of Aminopeptidase A and Aminopeptidase N by New β-Amino Thiols

Eric N. Chauvel,† Catherine Llorens-Cortès,‡ Pascale Coric,† Sherwin Wilk,§ Bernard P. Roques,*,† and Marie-Claude Fournié-Zaluski†

Unité de Pharmacochimie Moléculaire et Structurale, U266 INSERM – URA D 1500 CNRS, Université René Descartes, UFR des sciences pharmaceutiques et biologiques, 4, avenue de l'Observatoire, 75270 Paris Cedex 06, France, Collège de France, Chaire de médecine expérimentale, U36 INSERM, 3 rue d'Ulm, 75005 Paris, France, and Department of Pharmacology, Mount Sinaï School of Medecine, University of New York, New York, New York 10029

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Aminopeptidase A (APA) is a highly selective peptidase, which cleaves the N-terminal Glu or Asp residues of biologically active peptides, and has therefore been proposed to be involved in angiotensin II and CCK_8 metabolism. Highly potent and selective APA inhibitors are consequently required to study the physiological regulation of these two peptides. Using, as a model, Glu-thiol (4-amino-5-mercaptopentanoic acid), which was the first efficient APA inhibitor described but is however equipotent on APA (0.14 μM) and aminopeptidase N (APN) (0.12 μM), several β-amino thiol inhibitors have been synthesized. In these molecules, the length of the side chain was varied and the carboxylate group of Glu-thiol was replaced by other negatively charged groups, such as phosphonate, sulfonate, hydroxamate, and thiol. The inhibitory potency of one of these compounds, 22b (*S*)-3-amino-4-mercaptobutanesulfonate, was found to be nearly 100-fold better for APA than for APN, with an affinity (0.29 μM) almost equivalent to that of Glu-thiol. Hence, this compound is the first selective APA inhibitor reported, and as such, it should be an interesting probe to explore the physiological involvement of APA in the metabolism of neuropeptides like angiotensin II and CCK_8.

Introduction

Aminopeptidase A (APA, glutamyl aminopeptidase, EC 3.4.11.7) is thought to be responsible for the transformation of angiotensin II to angiotensin III, by hydrolyzing its N-terminal aspartyl residue.[1,2] The enzyme is highly selective for acidic amino acids[3] and is also probably involved in the metabolism of other peptides containing an N-terminal Asp or Glu residue, such as CCK_8.[4,5] Aminopeptidase N (APN, EC 3.4.11.2) seems to be the enzyme that inactivates angiotensin III, by cleaving its N-terminal arginyl residue,[6] and has been proposed to sequentially degrade CCK_7.[4] The relative physiological importance of peptides issued from angiotensin I processing in the central nervous system is controversial, but there is increasing evidence that angiotensin III is a crucial component of the brain renin angiotensin system.[2,7–9] However, in peptidergic cascades, where different peptidases sequentially release different putative active ligands from a single inactive precursor, the key to analyzing the various steps is to possess highly specific and potent inhibitors of each enzyme. The pharmacological responses measured in the presence of selective inhibitors can then be unequivocally related to the physiological action of tonically released peptides, as demonstrated for the opioid peptides, enkephalins, and the atrial natriuretic peptide (for a review, see ref 10). For the renin angiotensin system, this strategy was attempted but the inhibitors used did not have the necessary selectivity. Thus, the two inhibitors that have been most commonly used for APA and APN inhibition are amastatin and bestatin, respectively.[6,8,11] However, although amastatin was initially described as a specific APA inhibitor,[12] it is in fact more potent on APN and is even a better inhibitor of the latter enzyme than bestatin[13] which is a nonspecific inhibitor of various aminopeptidases.[14]

APA and APN are two enzymes belonging to the family of zinc metallopeptidases[15,16] with significant homologies in their amino acid sequences[17–20] and similarities in their physical properties.[21,22] Like neutral endopeptidase 24.11 (NEP)[15] and angiotensin converting enzyme (ACE),[23] APA and APN are characterized by a large glycosylated extracellular domain which contains the active site. On the basis of the structure and mechanism of action of the zinc metallopeptidase thermolysin,[24] efficient inhibitors can be obtained by introducing a zinc-coordinating group on molecules able to recognize the enzyme's S_1–S'_2 subsites (for reviews, see refs 10, 25, and 26). Potent and selective inhibitors of APN belonging to the series of mercaptans have been designed, some of them exhibiting K_i values in the 10 nM range.[27,28] In the case of APA, the best reported inhibitor, Glu-thiol,[29] which has a K_i value of 0.14 μM, is however equipotent on APN. There is therefore a need for potent specific inhibitors of APA. We have previously introduced various modifications in Glu-thiol, such as, for instance, replacement of its aliphatic side chain by phenyl or cyclohexyl moieties. This led to increased selectivity but reduced affinity for APA.[30]

In this study, we have introduced new modifications in the side chain of Glu-thiol, which was extended, to increase the selectivity versus APN and the affinity for APA, keeping in mind the respective substrate specificities of these enzymes. Moreover, the carboxylate group was replaced by other negatively charged groups. The results show that a selectivity factor for APA around 100

* To whom correspondence should be addressed. Tel: (33) 43-25-50-45. FAX: (33) 43-26-69-18.
† Université René Descartes.
‡ Collège de France.
§ University of New York.
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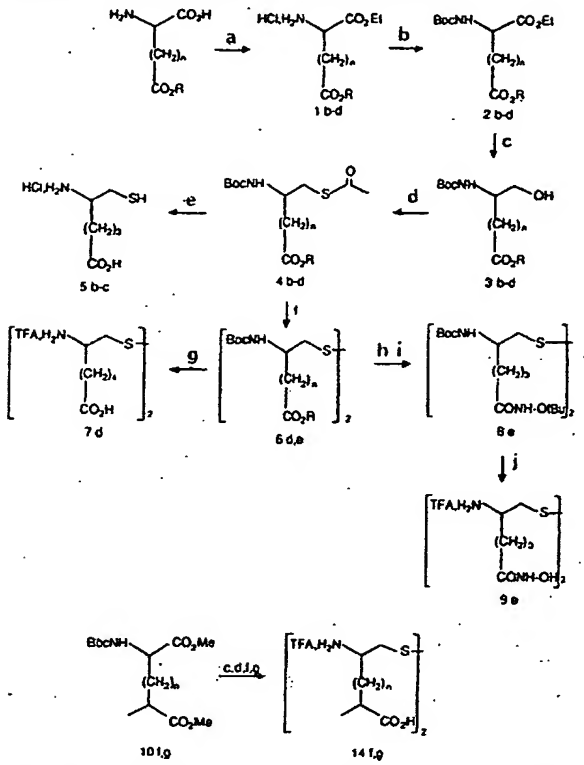
Scheme 1. Synthesis of the Carboxylates[a]



[a] (a) $SOCl_2$, EtOH, Δ; (b) $(Boc)_2O$; (c) $NaBH_4$; (d) PPh_3, $(iPrOOCN{=})_2$, CH_3COSH; (e) 6 N HCl, Δ; (f) NaOH, I_2; (g) TFA; (h) NaOH; (i) H_2NOtBu, DCC, HOBt; (j) BTFA/TFA.

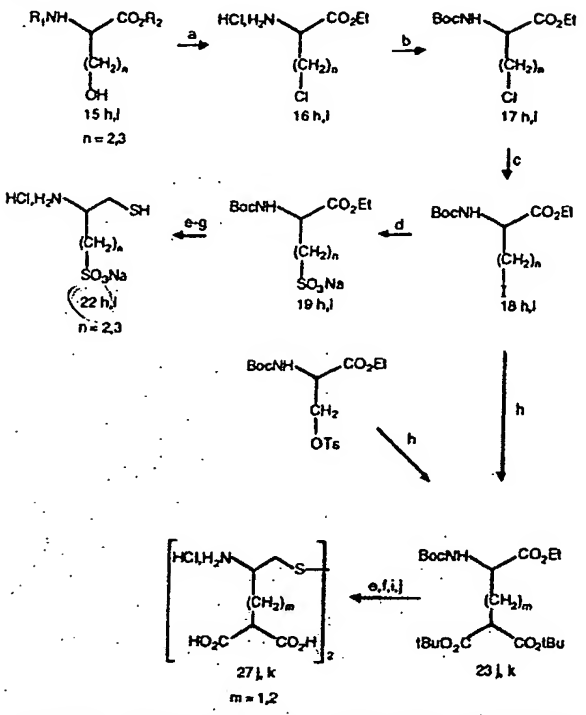
can be obtained in a compound, **22h**, (*S*)-3-amino-4-mercaptobutanesulfonate, with an inhibitory potency of 0.29 μM.

Results

Chemistry. The synthesis of the carboxylate-bearing compounds is summarized in Scheme 1. The starting materials were the commercially available 2-amino dicarboxylic acids, with or without protected side chains. After esterification of the carboxylate(s) (**1b–d**) and protection of the α-amino group (**2b–d**), the next two steps were the selective reduction of the α-ester into an alcohol (**3b–d**) as previously described,[30] using $NaBH_4$ in EtOH or EtOH/THF solutions, and the replacement of the alcohol by a thioester using the Mitsunobu reaction[31] (**4b–d**). The resulting compounds were deprotected, either directly by refluxing with 6 N HCl (**5b–c**) or in two steps by saponification and isolation of the disulfide (**6d,e**), followed by TFA treatment (**7d**). Compound **37n** was synthesized in the same way using dimethyl *N*-Boc-glutamate as starting material, except that the reduction was extended to both ester groups. The substitution of the two hydroxyls of **35n** by thioacetyl groups led to **36n**, and the final deprotection by 6 N HCl gave the desired compound. The hydroxamate **9e** was obtained from the disulfide **6e** by a coupling reaction with *O-tert*-butylhydroxylamine, and compound **8e** was then deprotected using a solution of boron tris(trifluoroacetate) (BTFA). The methyl-substituted compounds **14f,g** were obtained through reactions similar to those described for **7d**, starting from the Boc-amino diesters **10f,g**.

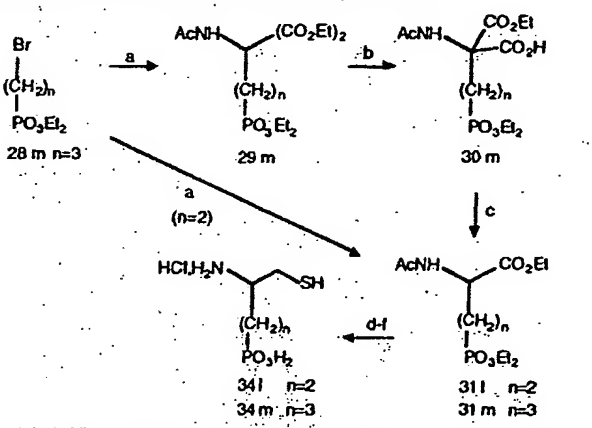
The synthesis of the sulfonates **22h,i** and the malonates **27j,k** is described in Scheme 2. From the hydroxyl α-amino acids **15h,i** the esterification in acidic conditions allowed the simultaneous substitution of the hydroxyl by a chloride to be performed (**16h,i**). After protection of the amino group (**17h,i**) and halide exchange by sodium iodide in acetone, a nucleophilic substitution of the halide by Na_2SO_3 or di-*tert*-butyl malonate led to the Boc-amino sulfonates **19h,i** or to the malonate derivatives **23j,k**. The subsequent steps of the synthesis, similar to those described in Scheme 1, gave the corresponding β-amino thiols **22h,i** and **27j,k**.

Scheme 2. Synthesis of the Sulfonates and the Dicarboxylates[a]



[a] (a) $SOCl_2$, EtOH, Δ; (b) $(Boc)_2O$; (c) NaI, acetone; (d) Na_2SO_3, dioxane/H_2O; (e) $NaBH_4$; (f) PPh_3, $(iPrN{=})_2$, CH_3COSH; (g) 6 N HCl, Δ; (h) $CH_2(CO_2tBu)_2$, NaH; (i) NaOH, I_2; (j) HCl, EtOAc.

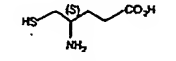
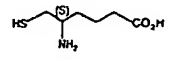
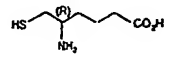
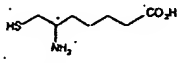
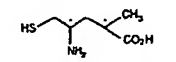
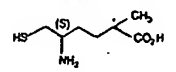
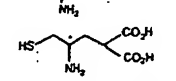
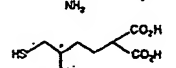
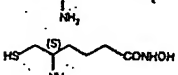
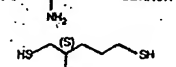
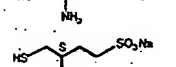
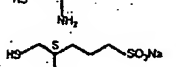
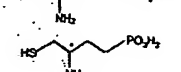
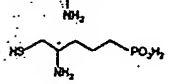
Scheme 3. Synthesis of the Phosphonates[a]



[a] (a) $AcNHCH(CO_2Et)_2$, EtONa; (b) KOH; (c) xylene, Δ; (d) $NaBH_4$; (e) PPh_3, $(iPrOOCN{=})_2$, CH_3COSH; (f) 6 N HCl, Δ.

Scheme 3 depicts the synthesis of the phosphonates. Malonate alkylation of diethyl bromoalkylphosphonates followed by monosaponification and decarboxylation led to the amino phosphonoesters **31l,m** that, after the

Table 1. Inhibitory Potencies of Various β-Amino Thiols for APA and APN[a]

Nb	Formula	Ki (μM)[a] APA	APN
a		0.14 ± 0.06	0.12 ± 0.02
5b		0.13 ± 0.08	0.33 ± 0.05
5c		0.98 ± 0.2	15.5 ± 5.7
7d		1.6 ± 0.06	0.28 ± 0.01
14f		4.1 ± 0.6	2.8 ± 0.3
14g		0.87 ± 0.4	4.0 ± 0.5
27j		3.5 ± 0.4	4.5 ± 0.4
27k		0.28 ± 0.04	0.60 ± 0.04
9e		2.0 ± 0.5	0.037 ± 0.009
37n		2.4 ± 0.6	0.032 ± 0.004
22h		0.29 ± 0.06	25 ± 11
22i		0.37 ± 0.02	1.9 ± 0.2
34l		0.39 ± 0.1	12 ± 2
34m		0.51 ± 0.03	3.7 ± 0.4

[a] The K_i values are the mean ± SEM of three independent experiments performed in triplicate.

steps described in Scheme 1, gave the phosphono β-amino thiols **34l,m**.

The synthetic pathway, leading to β-amino thiols from optically pure α-amino acids, retains the chirality of the starting material. Nevertheless, this was verified in the synthesis of the sulfonates **22h,i** which were obtained from the optically pure α-amino acids **15h,i**, as the introduction of the sulfonate moiety required a reflux in an aqueous solution of Na_2SO_3 which might be responsible for a racemization of the asymmetric carbon. The thioester of the fully protected intermediate **21h** (see the Experimental Section) was saponified under an inert atmosphere, and the free mercapto group obtained was derivatized using the chiral acyl chloride (S)-phenylbutyryl chloride. The resulting compound showed a single peak on HPLC, and a 2D NMR experiment confirmed the presence of only one stereoisomer.

Inhibitory Potencies. The results of the inhibition of APA by the Glu-thiol derivatives are summarized in Table 1. The extension of the lateral chain of this compound by one methylene group (**a**, **5b**) led to the same inhibition profile as that of the parent molecule. However, the addition of another methylene (**7d**) gave a significant loss in affinity (K_i value = 1.6 μM). Introduction of a methyl at the end of the side chain increased the K_i by a factor of 12 for the analog of Glu-thiol **14f** and only by a factor of 6 for the analog of homoGlu-thiol **14g**. Introduction of two carboxyl groups at the end of the side chain led to a 20-fold loss in affinity for **27j** and to the same potency (0.28 μM) for **27k** when compared to their monosubstituted counterparts. The hydroxamate **9e** had a 15-fold lower potency than homoGlu-thiol **5b**, whereas the dithiol **37n** was 18 times less active than **5b**. The sulfonates **22h,i** and the phosphonates **34l,m** had about the same activity as the corresponding carboxylates. For APN, there was a general loss in inhibitory potency when compared to Glu-thiol. Two exceptions were the hydroxamate **9e** and the dithiol **37n**, which were good inhibitors of APN with K_i values around 35 nM. Compounds **5b** and **7d** retained the same potency on APN as their Glu-thiol counterparts.

Discussion

APA is a selective enzyme, cleaving efficiently only N-terminal acidic residues.[3] When tested for arylamidase activity, it had a 4–5-fold preference for Glu-β-naphthylamide over Asp-β-naphthylamide.[32,33] This was the basis for the development of the APA inhibitor Glu-thiol, which was also shown to be 10-fold more active than Asp-thiol in inhibiting APA (K_i values = 0.14 and 1.2 μM, respectively).[29] We have previously investigated the active site of APA by modifying the α-functions of Glu-thiol and introducing a benzene ring or a cyclohexyl in the side chain of the inhibitor.[30] This showed that APA is characterized by relatively large catalytic and S_1 subsites and that the strength of enzyme inhibition by a compound bearing an aromatic side chain is dependent on the position of the carboxylate group on the cycle.

This time, in order to enhance the fit with the S_1 subsite of APA and the selectivity toward APN, we concentrated on two points in the inhibitor: the determination of the optimal length of an aliphatic P_1 moiety and the best negatively charged group to substitute for the carboxylate. As shown in Table 1, the homoGlu-thiol **5b** has the same K_i value for APA as Glu-thiol **a** but shows a 3-fold loss of activity for APN, therefore leading to an increase in selectivity, without any change in affinity. A further increase in side chain length, by the addition of a methylene group in **7d**, results in a 6-fold loss of inhibitory potency for APA (for the best enantiomer), while the K_i value for APN is similar to that found with Glu-thiol. The optimal length of the side chain for APA recognition therefore seems to be three methylenes, as in homoGlu-thiol **5b**. The introduction of substituents in the side chain of Glu-thiol and homoGlu-thiol gave some unexpected results. The presence of a methyl group in **14f** or of a hydrophilic carboxylate in **27j** induced a large loss of potency on both enzymes. However, the presence of the same substituents in the side chain of homoGlu-thiol (compounds **14g** and **27k**) led to different results since the K_i values for APA were not significantly modified and an improvement in APN inhibition was restricted to **14g**.

These results seem to indicate that not only the length of the side chain but a precise disposition of the carboxylate are important for optimal binding to APA, confirming our previous data with substituted aromatic side chains.[30]

Furthermore, compounds **27j,k** were synthesized to test the hypothesis of Danielsen et al.,[34] that the calcium

ion which activates APA could be positioned at the bottom of the S_1 subsite. Indeed, side chains bearing two carboxyl groups, such as in **27j,k**, have already been shown to bind Ca^{2+} with a good affinity in blood-clotting processes[35] and in calcium-binding proteins involved in mineralization.[36] However no increased inhibition of APA was observed with **27j,k**, which does not confirm the proposal of a Ca^{2+} ion located within the S_1 subsite.

The second part of this study was aimed at replacing the negatively charged carboxylate by other putatively deprotonated groups in the enzyme active site. When the carboxylate group of **5b** was changed, in **37n**, to a thiol or to an hydroxamate, in **9e**, the inhibitory potency decreased 18-fold for APA and increased 10-fold for APN, giving new selective and potent APN inhibitors. Interestingly, **9e** and **37n** differ from **5b** only by the strong decrease in the acidity of the thiol and hydroxamate groups which have replaced the carboxyl group. This suggests that a decrease in the pK_a of the side chain component modifies the binding to both enzymes. The side chain carboxylate was therefore replaced by a sulfonate or a phosphonate, with the aim of enhancing APA specificity. As expected, a selectivity factor around 100 was obtained for **22h**, together with a K_i value for APA (0.29 μM) very similar to that of Glu-thiol. The same result was observed for **34l**, but its selectivity was less pronounced (factor 31). It is interesting to note that the increased selectivity of **22h** and **34l** is not due to an enhanced recognition of APA but to a loss of potency on APN.

The addition of a methylene group in the side chains of **22h** and **34l** led to compounds **22i** and **34m** which exhibited similar inhibitory potencies toward APA but an improvement in APN active site recognition. These data are in apparent conflict with the propyl being the best side chain length, as determined with the carboxylate-containing inhibitors. This suggests that, due to the increase in size and the decrease in pK_a of the phosphonate and sulfonate moieties as compared to the side chain carboxylate, these groups exert a more important repulsive interaction in the S_1 subsite of APN at the end of an ethyl rather than a propyl side chain.

Taking into account the selectivity and affinity displayed by **22h**, this compound is, as far as we know, the best existing selective APA inhibitor. It will therefore be an interesting tool to study the diverse biological roles of APA (for a review, see ref 5).

In conclusion, the exploration of the S_1 subsite of APA presented here confirms our previous results, showing that the positioning of the negative group at the end of the side chain of a β-amino thiol inhibitor is critical for optimal enzyme recognition.[30] Having reached a reasonable selectivity, the next step is now to increase affinity for APA. One possibility could be to extend the occupation of the active site. Preliminary work with di- and tripeptides, with the aim of developing pseudopeptide inhibitors that would interact with the S_1, S_1', and S_2' subsites of APA, is encouraging.

Experimental Section

Inhibitory Potency. Aminopeptidase A, purified from rabbit kidney,[29] hydrolyzed approximately 100 μmol $mL^{-1}·h^{-1}$ of the substrate α-L-glutamyl-β-naphthylamide (GluNA). GluNA (K_m = 130 μM) was from Bachem (Bubendorf, Switzerland). Aminopeptidase N from hog kidney was purchased from Boehringer Mannheim (Meylan, France) and suspended in 3.2 M ammonium sulfate, 50 mM Tris buffer, pH 7.4. [^{3}H]-Tyr^1-Leu^5-enkephalin (30 Ci/mmol) was from Amersham. The solutions of thiol inhibitors were prepared in 50 mM Tris buffer, pH 7.4, containing dithiothreitol (DTT, 100 equiv/equiv of inhibitor). K_i values were determined from IC_{50}s, assuming a competitive inhibition, using the Chen–Prusoff relationship.

Aminopeptidase A. The procedure of Goldbarg[37] was used with a downscale modification on a microplate. APA was incubated for 1 h at 37 °C, with or without increasing concentrations of inhibitors and with 200 μM GluNA, in a total volume of 100 μL in 50 mM Tris-HCl buffer, pH 7.4, with 4 mM $CaCl_2$. The reaction was stopped by adding 10 μL of 3 N HCl; 25 μL of 87 mM (0.6%) $NaNO_2$ was then added to each well followed 3 min later by 50 μL of 0.13 M (1.5%) ammonium sulfamate. After a further 5 min, 25 μL of a 23 mM (0.6%) solution of *N*-(1-naphthyl)ethylenediamine dihydrochloride in 95% EtOH was added and the plate was incubated for 30 min at 37 °C. The absorbance was measured at 560 nm.

Aminopeptidase N. APN was preincubated for 15 min at 25 °C with or without increasing concentrations of inhibitors, in a total volume of 100 μL in 50 mM Tris-HCl buffer, pH 7.4. [^{3}H]Tyr^1-Leu^5-enkephalin (K_m = 50 μM) was added to a final concentration of 10 nM, and the reaction was stopped after 15 min by adding 10 μL of 0.5 M HCl. The tritiated metabolite [^{3}H]Tyr was separated on polystyrene beads as described by Vogel and Altstein,[38] and the radioactivity was measured by liquid scintillation counting.

Chemistry. Amino acids were obtained from Bachem (Bubendorf, Switzerland). Homoserine, homoglutamic acid, and all the other reagents were obtained from Aldrich (Saint Quentin Fallavier, France) unless otherwise stated. The solvents were from Merck (Nogent sur Marne, France).

Melting points of the crystallized compounds were measured on an electrothermal apparatus and are reported uncorrected. Chromatography was carried out with Merck silica gel (230–400 mesh). TLC was performed on precoated silica gel plates (60F-254, 0.2 mm thick, Merck) with the following solvent systems (v/v): A1, CH_2Cl_2:MeOH, 9:1; A2, CH_2Cl_2:MeOH, 8:2; B1, *n*-hexane:EtOAc, 6:4; B2, *n*-hexane:EtOAc, 4:1; C, CH_2Cl_2:MeOH:AcOH, 9:1:0.5; D, *n*-hexane:EtOAc:AcOH, 5:5:0.5; E, $CHCl_3$:MeOH:H_2O:AcOH, 5:5:1:0.5; F, *n*-BuOH:H_2O:AcOH, 4:2:2. Plates were developed with UV light, iodine vapor, or ninhydrin. The purity of the final compounds was also checked by HPLC using a silica column (Touzart & Matignon, Vitry sur Seine, France) with CH_2Cl_2–MeOH–AcOH as solvent. The eluted peaks were monitored at 236 nm. The structure of the compounds was confirmed by ^{1}H NMR spectroscopy on a Bruker AC spectrometer (270 MHz) in DMSO-d_6 using HMDS as internal reference, and satisfactory analyses (<±0.4%) were obtained (C, H, N) for all compounds.

The following abbreviations are used, MeOH, methanol; EtOH, ethanol; EtOAc, ethyl acetate; THF, tetrahydrofuran; Boc, *tert*-butyloxycarbonyl; Boc_2O, di-*tert*-butyl dicarbonate; Et_2O, diethyl ether; DME, 1,2-dimethoxyethane; DMF, *N,N*-dimethylformamide; TFA, trifluoroacetic acid; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; LDA, lithium diisopropylamide.

General Procedure for Protection of the Amino Group. Procedure A. The amino group was protected with a *tert*-butyloxycarbonyl group using the previously described method carried out in DMF.[39]

General Procedures for Esterification of the Carboxylate Group. Procedure B. The methyl or ethyl esters were prepared in acidic conditions by the Fischer method (alcohol + $SOCl_2$).

General Procedures for the Reduction of the Ester Function. Procedure C.1. The Boc-amino diester was dissolved in EtOH:water (1:1) (3 mL/mmol), and $NaBH_4$ (1 equiv) in the same solvent was added dropwise at 0 °C. After 15 min, the mixture was heated at 50 °C and stirred for 2–4 h. The EtOH was then evaporated and the resulting solution extracted with EtOAc, washed with brine, dried over Na_2SO_4, and evaporated to dryness.

Procedure C.2. The Boc-amino diester (1 equiv) was dissolved in dry EtOH:THF (8 mL/mmol) and cooled to 0 °C. $NaBH_4$ (4 equiv) and LiCl (4 equiv) in solution in the same

solvent were added dropwise at 0 °C. The mixture was then allowed to warm to room temperature and stirred overnight. The reaction was stopped with 1 N HCl, and the mixture was extracted with EtOAc, washed with water, 1 N HCl, $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated in vacuo.

General Procedure for Substitution of the Hydroxyl Group. Procedure D: Thioacetylation via the Mitsunobu Reaction.[31] Triphenylphosphine (2 equiv) was dissolved in dry THF (3.5 mL/mmol). At 0 °C, diisopropyl azodicarboxylate (2 equiv) was added and the mixture stirred for 30 min until a light yellow precipitate was formed. The alcohol (1 equiv) dissolved in THF (3 mL/mmol), and CH_3COSH (2 equiv) were added. The temperature was allowed to rise slowly to room temperature while the mixture was stirred overnight. After evaporation in vacuo, the residue was dissolved in EtOAc and washed successively with a 10% $NaHCO_3$ solution, H_2O, and brine before being dried over Na_2SO_4. After evaporation, *n*-hexane/EtOAc was added to the residue and the precipitate eliminated. The filtrate was evaporated and the residue purified by flash chromatography on a silica gel column, using *n*-hexane:EtOAc, 4:1, as eluent.

General Procedures for Deprotection Reactions. Procedure E.1: Saponification of Esters and Thioesters. The product (1 equiv) was dissolved in its corresponding alcohol (EtOH or MeOH) (5 mL/mmol), and 1 N NaOH (2.5 equiv) was added at 0 °C. The mixture was stirred for 30 min at 0 °C and for 3 h at room temperature. A solution of I_2 in EtOH was added until a persistant yellow color was obtained. The excess iodine was reduced by $Na_2S_2O_3$, and the solution was evaporated in vacuo. The residue was taken up in water, acidified with 3 N HCl, and extracted with EtOAc. The organic layer was then washed with $Na_2S_2O_3$, H_2O, and brine, dried over Na_2SO_4, and evaporated to dryness.

Procedure E.2: Deprotection of *N*-Boc and *tert*-Butyl Ester Groups by TFA. The product (1 equiv) was dissolved in CH_2Cl_2 (3 mL/mmol). Anisole (1 equiv) and TFA (10 equiv) were then added at 0 °C. After the mixture had stirred for 30 min at 0 °C and for 2 h at room temperature, c-hexane was added to facilitate the evaporation of the TFA in vacuo. The residue was taken up with c-hexane and evaporated to dryness three or four times.

Procedure E.3: General Deprotection by Refluxing in 6 N HCl. The product was taken up in a large excess of 6 N HCl, and the mixture was heated at 130 °C and stirred overnight. The mixture was then allowed to cool down, before being evaporated to dryness. The residue was dissolved in H_2O and reevaporated. This process was repeated three or four times to eliminate excess acid before lyophilization.

Procedure E.4: Deprotection by HCl in EtOAc. A 2.7 N solution of HCl in EtOAc (1 mL/mmol) was added at 0 °C to the compound solubilized in EtOAc. The mixture was then stirred for 2 h at room temperature before evaporation to dryness.[40]

General Procedure for Halide Exchange. Procedure F. The chloro derivative was added to a solution of dry NaI (2 equiv), in dry acetone (1 mL/mmol). The mixture was heated to reflux under a N_2 atmosphere for 8 h. After cooling, the mixture was filtered off and the filtrate evaporated to dryness. It was then taken up in EtOAc, washed with Na_2SO_3, H_2O, and brine, dried over Na_2SO_4, and evaporated to dryness to yield the iodo derivative that was used without purification.

Most of the physical data of the compounds synthesized are summarized in Table 2. Additional data about the end products and some intermediates follow.

(*S*)-5-Amino-6-mercaptohexanoic Acid, Hydrochloride (5b). (*S*)-2-Aminoadipic acid was esterified following procedure B and gave a white solid (100%), mp 118–119 °C, R_f(A1) 0.32. The amino group of the diethyl ester obtained was protected by a Boc group (procedure A) (100%), oily compound, R_f(B1) 0.48. The reduction of the α-ester was performed by procedure C.1. A white solid was obtained (67%), mp 45 °C, R_f(A1) 0.42. The thioacetylation was performed by procedure B. An oily product was isolated (80%), R_f(B1) 0.56. Treatment by TFA (procedure E.2) gave the titled compound (81%): mp 115–116 °C, R_f(E) 0.59; HPLC CH_2Cl_2:MeOH:AcOH, 7.5:2.5:0.1, t_R 8.3 min; $[\alpha]_D^{22} = +17.1°$ in H_2O, $c = 1.035$; ^1H NMR (DMSO) δ 1.52 (m, 4 H, CH_2CH_2CH), 2.2 (t, 2 H, CH_2CO), 2.70 (m, 2 H, CH_2S), 2.82 (d, 1H, SH), 3.15 (m, 1 H, CH), 8.15 (s, 3 H, NH_3^+), 12 (s, 1 H, CO_2H). Anal. ($C_6H_{13}NO_2S$·HCl) C, H, N.

The enantiomers (*S*)-**5b** and (*R*)-**5c** were synthesized by the same pathway. They had the same physical constants excepted for **5c**: $[\alpha]_D^{22} = -17.3°$ in H_2O, $c = 1.039$. Anal. ($C_6H_{13}NO_2S$·HCl) C, H, N.

7-*tert*-Butyl 1-Ethyl 2(*R,S*)-Aminopimelate (1d). 5-Bromovaleric acid (9.49 g, 52 mmol) was esterified with isobutylene using the procedure described by Mokotoff[41] to obtain *tert*-butyl 5-bromopentanoate as a colorless oil, 11.64 g (94%), R_f(B1) 0.64.

Alkylation of *tert*-butyl 5-bromopentanoate (5 g, 21 mmol) was performed using the procedure described by Duhamel,[42,43] with ethyl(diphenylmethylene)glycinate and LDA. Purification by flash chromatography in *n*-hexane:EtOAc (6:1) gave 7-*tert*-butyl 1-ethyl 2-[*N*-(diphenylmethylene)amino]pimelate as a yellow oil, 1.964 g (26%), R_f(B2) 0.38. The diphenylimine group of 7-*tert*-butyl 1-ethyl 2-[*N*-(diphenylmethylene)amino]-pimelate (1.93 g, 4.6 mmol) was cleaved using 1 N HCl in Et_2O[44] to give **1d** as a yellow oil, 500 mg (42%), R_f(A1) 0.34.

7,7′-Dithiobis(6(*R,S*)-aminoheptanoic acid), Bis(trifluoroacetate) (7d). HPLC CH_2Cl_2:MeOH:AcOH, 7.5:2.5:0.1, t_R 6.5 min; ^1H NMR (DMSO + TFA) δ 1.4, 1.54, and 1.67 (3 m, 3 × 2 H, $CH_2CH_2CH_2CH$), 2.25 (t, 2 H, CH_2CO), 3.0 (m, 2 H, CH_2S), 3.4 (m, 1 H, CH), 7.96 (s, 3 H, NH_3^+). Anal. ($C_{14}H_{28}N_2O_4S_2$·2TFA) C, H, N.

6,6′-Dithiobis[5-*N*-Boc-(*S*)-amino)hexane[*N*-*tert*-butyloxy)carboxamide]] (8e). At 0 °C, to a solution of **6e** (368 mg, 0.7 mmol) in dry THF were added successively H_2NOtBu, HCl (2.1 equiv), Et_3N (2.2 equiv), HOBt, H_2O (2 equiv) in THF (6 mL), and DCC (2.4 equiv) in $CHCl_3$ (5 mL). The mixture was stirred for 15 min at 0 °C, and then it was allowed to warm to room temperature overnight. It was then filtrated, evaporated to dryness, taken up in EtOAc, and washed with H_2O, citrate 10%, H_2O, $NaHCO_3$ 10%, H_2O, and brine. It was then dried over Na_2SO_4, filtered, and evaporated to dryness. Purification by gel chromatography in CH_2Cl_2:MeOH, 9:1, gave **8e** as a colorless oil, 328 mg (70%), R_f(A1) 0.47.

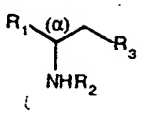
6,6′-Dithiobis[5-(*N*-Boc-(*S*)-amino)hexane(*N*-hydroxycarboxamide)] (9e). As described by Pless,[45] at 0 °C under Ar, **8e** (140 mg, 0.21 mmol) was stirred in TFA (0.5 mL); 1.7 mL of a solution of 1 M BTFA (Merck) in TFA was added. The mixture was stirred at 0 °C for 2 h. It was then evaporated to dryness at room temperature; c-hexane was added and the mixture evaporated to dryness several times. It led to **9e** as a light pink solid: 118 mg (97%), mp >260 °C, R_f(F) 0.53; HPLC CH_2Cl_2:MeOH:AcOH, 8.2:0.1, t_R 6.0 min; ^1H NMR (DMSO + TFA) δ 1.45–1.98 (m, 4 H, CH_2CH_2CH), 2.25 (t, 2 H, CH_2CO), 2.96 (m, 2 H, CH_2S), 3.64 (m, 1 H, CH), 7.96 (bs, 3 H, NH_3^+), 10.09 (s, 1 H, *NH*OH). Anal. ($C_{12}H_{28}N_4O_4S_2$·2TFA) C, H, N.

Methyl 4-(*N*-Boc-(*R,S*)-amino)-5-hydroxy-2(*R,S*)-methylpentanoate (11f). (2*R,S* 4*R,S*)-4-Methyl-*N*-Boc-glutamic acid dimethyl ester was synthesized using the procedure described by Done[46] followed by esterification (B) and amino protection (A). This compound, **10f** (3 g, 8 mmol), was reduced using procedure C.1 followed by purification by flash chromatography in CH_2Cl_2:MeOH (9:1) to afford **11f** as a yellow oil, 1.18 g (56%), R_f(A1) 0.40.

5,5′-Dithiobis(4(*R,S*)-amino-2(*R,S*)-methylpentanoic acid), Bis(trifluoroacetate) (14f). HPLC CH_2Cl_2:MeOH:AcOH, 8.2:0.1, t_R 5.9 min; ^1H NMR (DMSO + TFA) δ 1.07 (d, 3 H, CH_3CH), 1.56 and 1.97 (m, 2 H, CH_2), 2.58 (m, 1 H, CH_Y), 2.92 (m, 2 H, CH_2S), 3.38 (m, 1 H, CH_α), 7.9 (s, 3 H, NH_3^+), 12.2 (s, 1 H, CO_2H). Anal. ($C_{12}H_{24}N_2O_4S_2$·2TFA) C, H, N.

1-Methyl 6-Ethyl 2-*N*-Boc-(*S*)-amino)-5(*R,S*)-methyladipate (10g). Ethyl 2-(diethylphosphono)propanoate (1.6 equiv) and NaH (1.6 equiv) in dry DME were stirred at 0 °C for 15 min until *tert*-butyl 2-(*N*-Boc-amino)-4-oxobutanoate (2.94 g, 10.8 mmol), prepared according to the procedure of Ramsamy et al.,[47] was added. The mixture was then heated to reflux for 5 h and evaporated to dryness. It was taken up in EtOAc, washed with water and brine, dried over Na_2SO_4,

Table 2. Physical Constants of the Compounds Synthesized[a]



compd	R_1	R_2	R_3	Cα	method	yield (%)	mp (°C)	TLC
1b	CO_2Et	H, HCl	$(CH_2)_2CO_2Et$	S	B	quant	118	0.71 (E)
2b	CO_2Et	Boc	$(CH_2)_2CO_2Et$	S	A	quant	oil	0.48 (B1)
3b	CH_2OH	Boc	$(CH_2)_2CO_2Et$	S	C.1	67	45	0.42 (A1)
4b	CH_2SAc	Boc	$(CH_2)_2CO_2Et$	S	D	80	oil	0.56 (B1)
5b	CH_2SH	H, HCl	$(CH_2)_2CO_2H$	S	E.3	81	115	0.59 (E)
2d	CO_2Et	Boc	$(CH_2)_3CO_2tBu$	*	A	52	oil	0.64 (B1)
3d	CH_2OH	Boc	$(CH_2)_3CO_2tBu$	*	C.2	91	oil	0.59 (A1)
4d	CH_2SAc	Boc	$(CH_2)_3CO_2tBu$	*	D	76	oil	0.68 (B1)
6d	CH_2S-I_2	Boc	$(CH_2)_3CO_2tBu$	*	E.1	61	oil	0.63 (B1)
7d	CH_2S-I_2	H	$(CH_2)_3CO_2^-, NH_4^+$	*	E.1	61	oil	0.63 (B1)
6e	CH_2S-I_2	Boc	$(CH_2)_2CO_2H$	*	E.1	61	oil	0.63 (B1)
12f	CH_2SAc	Boc	$CH(CH_3)CO_2Me$	*	D	42	oil	0.50 (B1)
13f	CH_2S-I_2	Boc	$CH(CH_3)CO_2H$	*	E.1	82	oil	0.44 (D)
14f	CH_2S-I_2	H, TFA	$CH(CH_3)CO_2H$	*	E.2	51	99	0.20 (E)
11g	CH_2OH	Boc	$CH_2CH(CH_3)CO_2Et$	S	C.1	50	oil	0.49 (A1)
12g	CH_2SAc	Boc	$CH_2CH(CH_3)CO_2Et$	S	D	53	oil	0.51 (B1)
19g	CH_2S-I_2	Boc	$CH_2CH(CH_3)CO_2H$	S	E.1	71	oil	0.42 (D)
14g	CH_2S-I_2	H, TFA	$CH_2CH(CH_3)CO_2H$	S	E.2	57	132	0.38 (E)
16h	CO_2Et	H, HCl	CH_2Cl	S	B	98	105	0.36 (A1)
17h	CO_2Et	Boc	CH_2Cl	S	A	82	55	0.61 (B1)
18h	CO_2Et	Boc	CH_2I	S	F	80	49	0.61 (B1)
19h	CO_2Et	Boc	CH_2SO_3Na	S	*b*	78	100	0.28 (A2)
20h	CH_2OH	Boc	CH_2SO_3Na	S	C.1	75	oil	0.12 (A2)
21h	CH_2SAc	Boc	CH_2SO_3Na	S	D	80	oil	0.20 (A2)
22h	CH_2SH	H, HCl	CH_2SO_3Na	S	E.3	quant	>260	0.23 (F)
16i	CO_2Et	H, HCl	$(CH_2)_2Cl$	S	B	92	oil	0.38 (A1)
17i	CO_2Et	Boc	$(CH_2)_2Cl$	S	A	52	oil	0.40 (B2)
18i	CO_2Et	Boc	$(CH_2)_2I$	S	F	55	oil	0.37 (B2)
19i	CO_2Et	Boc	$(CH_2)_2SO_3Na$	S	*b*	quant	150	0.24 (A2)
20i	CH_2OH	Boc	$(CH_2)_2SO_3Na$	S	C.2	51	92	0.10 (A2)
21i	CH_2SAc	Boc	$(CH_2)_2SO_3Na$	S	D	85	101	0.22 (A2)
22i	CH_2SH	H, HCl	$(CH_2)_2SO_3Na$	S	E.3	quant	157	0.39 (F)
23j	CO_2Me	Boc	$CH(CO_2tBu)_2$	*	*c*	60	oil	0.63 (B1)
24j	CH_2OH	Boc	$CH(CO_2tBu)_2$	*	C.2	70	oil	0.35 (B1)
25j	CH_2SAc	Boc	$CH(CO_2tBu)_2$	*	D	55	oil	0.60 (B1)
26j	CH_2S-I_2	Boc	$CH(CO_2tBu)_2$	*	E.1	82	oil	0.66 (B1)
27j	CH_2S-I_2	H, HCl	$CH(CO_2H)_2$	*	E.4	66	211	0.41 (F)
23k	CO_2Et	Boc	$CH_2CH(CO_2tBu)_2$	*	*c*	63	oil	0.71 (B1)
24k	CH_2OH	Boc	$CH_2CH(CO_2tBu)_2$	*	C.2	86	oil	0.25 (B1)
25k	CH_2SAc	Boc	$CH_2CH(CO_2tBu)_2$	*	D	56	oil	0.66 (B1)
26k	CH_2S-I_2	Boc	$CH_2CH(CO_2tBu)_2$	*	E.1	86	oil	0.73 (B1)
27k	CH_2S-I_2	H, HCl	$CH_2CH(CO_2H)_2$	*	E.4	97	124	0.52 (F)
31l	CO_2Et	Ac	$CH_2PO_3Et_2$	*	*d*	86	oil	0.58 (C)
32l	CH_2OH	Ac	$CH_2PO_3Et_2$	*	C.2	93	oil	0.24 (A1)
33l	CH_2SAc	Ac	$CH_2PO_3Et_2$	*	D	38	oil	0.48 (A1)
34l	CH_2SH	H, HCl	$CH_2PO_3H_2$	*	E.3	quant	oil	0.26 (F)
29m	$(CO_2Et)_2$	Ac	$(CH_2)_2PO_3Et_2$	–	*d*	84	oil	0.58 (A1)
32m	CH_2OH	Ac	$(CH_2)_2PO_3Et_2$	*	C.2	quant	oil	0.31 (C)
33m	CH_2SAc	Ac	$(CH_2)_2PO_3Et_2$	*	D	46	oil	0.46 (A1)
34m	CH_2SH	H, HCl	$(CH_2)_2PO_3H_2$	*	E.3	quant	oil	0.29 (G)
35n	CH_2OH	Boc	$(CH_2)_2OH$	S	C.2	86	oil	0.25 (A1)
36n	CH_2SAc	Boc	$(CH_2)_2SAc$	S	D	27	76	0.57 (B1)
37n	CH_2SH	H, HCl	$(CH_2)_2SH$	S	E.3	98	oil	0.77 (E)

[a] Methods and TLC systems are described in the experimental section. * = *R,S*. [b] See ref 51. [c] See refs 52 and 53. [d] See ref 50.

and evaporated to dryness. Purification by flash chromatography in c-hexane:EtOAc (9:1) gave (*S*)-1-*tert*-butyl 6-ethyl 2-(*N*-Boc-amino)-5-methylhex-4-enedioate as a yellow oil, 1.768 g (46%), R_f(B2) 0.64.

(*S*)-1-*tert*-butyl 6-ethyl 2-(*N*-Boc-amino)-5-methylhex-4-enedioate (1.92 g, 5.4 mmol) was then dissolved in MeOH. Pt/C was added as a catalyst, and the mixture was stirred under an atmosphere of hydrogen overnight. After filtration of the catalyst, the remaining solution was evaporated to dryness and gave (2*S*,5*R*,*S*)-1-*tert*-butyl 6-ethyl 2-(*N*-Boc-amino)-5-methyladipate as a yellow oil, 1.6 g (83%), R_f(B2) 0.64.

Deprotection of (2*S*,5*R*,*S*)-1-*tert*-butyl 6-ethyl 2-(*N*-Boc-amino)-5-methyladipate (1.6 g, 4.5 mmol) was carried out with procedure E.4. (2*S*,5*R*,*S*)-6-Ethyl 2-amino-5-methyladipate was obtained as a colorless oil, 1.1 g (quantitative), R_f(E) 0.54.

(2*S*,5*R*,*S*)-6-Ethyl 2-amino-5-methyladipate (1.06 g, 4.4 mmol) was then amino protected (A) and purified by flash chromatography in CH_2Cl_2:MeOH:AcOH (9:0.3:0.5), giving (2*S*,5*R*,*S*)-6-ethyl 2-(*N*-Boc-amino)-5-methyladipate as a yellow oil, 900 mg (67%), R_f(A1) 0.39.

Finally, (2*S*,5*R*,*S*)-6-ethyl 2-(*N*-Boc-amino)-5-methyladipate (900 mg, 3 mmol) was esterified under basic conditions via the cesium salt of the acid and CH_3I[48] into 10g as a yellow oil, 790 mg (84%), R_f(A1) 0.42.

6,6'-Dithiobis(5(*S*)-amino-2(*R*,*S*)-methylhexanoic acid), Bis(trifluoroacetate) (14g). HPLC CH_2Cl_2:MeOH:AcOH, 8:2:0.1, t_R 5.8 min. ^1H NMR (DMSO) δ 1.05 (d, 3 H, CH_3CH), 1.4–1.6 (m, 4 H, CH_2CH_2CH), 2.3 (m, 1 H, $CHCOOH$), 2.95 (m, 2 H, CH_2S), 3.3 (m, 1 H, CH), 7.95 (s, 3 H, NH_3^+), 12.3 (s, 1 H, COOH). Anal. ($C_{14}H_{28}N_2O_4S_2$·2TFA) C, H, N.

Sodium 3(*S*)-Amino-4-mercaptobutanesulfonate (22h). By treatment with EtOH and $SOCl_2$, L-homoserine gave the intermediate **16h**, which was esterified on the α-carboxylate and halogenated on the side chain, white solid (98%), mp 105–106 °C, R_f(A1) 0.36.

After protection of the α-amine (procedure A, compound **17h**), the halide exchange was performed by procedure F, leading to **18h**, white solid (80%), mp 49 °C, R_f(B1) 0.61. The sulfonation was carried out as previously reported,[51] giving a yellow solid, **19h** (78%), R_f(A2) 0.28. The last steps of the synthesis, reduction of the α-ester (procedure C, compound **20h**), thioacetylation (procedure D, compound **21h**), and deprotection (procedure E.3), gave the compound desired: white solid; mp >260 °C; R_f(F) 0.23; $[\alpha]^{19}_D = +11.1°$ in H_2O, $c = 2.145$; HPLC CH_2Cl_2:MeOH:AcOH, 8:2:0.1, t_R 5.1 min; ^1H NMR (DMSO + TFA) δ 1.84–2.14 (m, 2 H, CH_2CH), 2.7 (m, 4 H, CH_2SH and CH_2SO), 3.32 (m, 1 H, CH), 7.97 (bs, 3 H, NH_3^+). Anal. ($C_4H_{10}NNaO_3S_2$·HCl) C, H, N.

(S)-*tert*-Butyl 2-(*N*-Boc-(S)-amino)-5-hydroxypentanoate (15i). To a solution of Boc-L-Glu(OH)OtBu (5.35 g, 17 mmol) in dry DME were successively added under N_2 at −15 °C 4-ethylmorpholine (1 equiv) and isobutyl chloroformate (1 equiv).[49] After 5 min, the precipitate of 4-ethylmorpholine, HCl was filtered off and washed by DME. Filtrate and washings were combined in a large flask in an ice–salt bath. A solution of $NaBH_4$ (1.5 equiv) in H_2O (8.5 mL) was added. After the solution was stirred for 15 min at the same temperature, H_2O (425 mL) was added next. Evaporation to dryness and purification in *n*-hexane:EtOAc, 6:4, gave **15i** as a colorless oil, 4.78 g (96%), R_f(A1) 0.45, R_f(B1) 0.35.

Sodium 4-Amino-5-mercaptopentanesulfonate (22i): $[\alpha]^{20}_D = -0.2°$ in H_2O, $c = 0.619$; HPLC CH_2Cl_2:MeOH:AcOH, 8:2:0.1, t_R 4.2 min; ^1H NMR (DMSO + TFA) δ 1.74 (m, 4 H, CH_2CH_2CH), 2.64 (m, 2 H, CH_2SO), 2.75 (m, 2 H, CH_2SH), 3.25 (m, 1 H, CH), 8.0 (bs, 3 H, NH_3^+). Anal. ($C_5H_{12}NNaO_3S_2$·HCl) C, H, N.

5,5'-Dithiobis(4(*R,S*)-amino-2-carboxypentanoic acid), Bis(hydrochloride) (27j): HPLC CH_2Cl_2:MeOH:AcOH, 8:2:0.1, t_R 5.3 min; ^1H NMR (DMSO + TFA) δ 2.25 and 2.48 (2 m, 2 × 1 H, CH_2CH), 2.78 and 2.85 (dd, 2 H, CH_2S), 3.18 (m, 1 H, CHγ), 3.82 (m, 1 H, CHα), 8.12 (bs, 3 H, NH_3^+). Anal. ($C_{12}H_{20}N_2O_8S_2$·2HCl) C, H, N.

6,6'-Dithiobis(5(*R,S*)-amino-2-carboxyhexanoic acid), Bis(hydrochloride) (27k). The intermediate **18h** (described in the preparation of **22h**) was treated with di-*tert*-butyl malonate and NaH, leading to **23k**. The reduction of the α-ester (procedure C.2) gave **24k** as an oily product (86%), R_f(B1) 0.25. The thioacetylation (procedure D) led to **25k**, oily product (56%), R_f(B1) 0.66. The saponification of the thioester (procedure E.1) and the deprotection of the *tert*-butyl groups (procedure E.4) gave the titled compound as a white solid: mp 124 °C; R_f(F) 0.52; HPLC CH_2Cl_2:MeOH:AcOH, 7.5:2.5:0.1, t_R 8.3 min; ^1H NMR (DMSO + TFA) δ 1.63 and 2.02 (m, 4 H, CH_2CH_2CH), 3.04 (m, 2 H, CH_2S), 3.29 (t, 1 H, CHδ), 3.42 (m, 1 H, CHα), 8.09 (bs, 3 H, NH_3^+). Anal. ($C_{14}H_{24}N_2O_8S_2$·2HCl) C, H, N.

Ethyl 2-(*N*-Acetyl-(*R,S*)-amino)-4-(diethylphosphono)-butanoate (31l). Diethyl (2-bromoethyl)phosphonate (25.5 g, 100 mmol) was alkylated using the procedure described by Chambers[50] with diethyl acetamidomalonate. **31l** was recovered decarboxylated in the aqueous phase as a yellow oil, 27 g (86%) (the yield is calculated for the decarboxylated product), R_f(C) 0.58. NMR data showed the product had undergone decarboxylation.

Sodium 3(*R,S*)-Amino-(4-mercaptobutyl)phosphonate (34l): HPLC CH_2Cl_2:MeOH:AcOH, 8:2:0.1, t_R 4.2 min; ^1H NMR (DMSO + TFA) δ 1.53–1.94 (m, 4 H, CH_2CH_2), 2.7 (m, 2 H, CH_2S), 3.25 (m, 1 H, CH), 7.97 (bs, 3 H, NH_3^+). Anal. ($C_4H_{12}NO_3PS$·HCl) C, H, N.

Sodium 4(*R,S*)-Amino-(5-mercaptopentyl)phosphonate (34m). HPLC CH_2Cl_2:MeOH:AcOH, 8:2:0.1, t_R 4.2 min; ^1H NMR (DMSO + TFA) δ 1.65 (m, 6 H, $CH_2CH_2CH_2$), 2.72 (m, 2 H, CH_2S), 3.22 (m, 1 H, CH), 7.95 (s, 3 H, NH_3^+). Anal. ($C_5H_{14}NO_3PS$·HCl) C, H, N.

2(*S*)-Aminopentane-1,5-dithiol, Hydrochloride (37n). The reduction of both ester functions of Boc-Glu(OCH_3)OCH_3 by procedure C.2 led to the diol **35n**, oily product (86%), R_f(A1) 0.25. The thioacetylation (procedure D) gave the intermediate **36n** as a white solid (27%), mp 76 °C, R_f(B1) 0.57. The deprotection of the three functional groups by procedure E.3 gave the titled compound as an oily product (98%): R_f(E) 0.77; HPLC CH_2Cl_2:MeOH:AcOH, 8:2:0.1, t_R 11.6 min; ^1H NMR ($CDCl_3$) δ 1.46 (t, 1 H, SH), 1.64 and 2.05 (m, 4 H, CH_2CH_2CH), 1.97 (t, 1 H, SH), 2.52 (m, 2 H, CH_2CH_2SH), 2.88 (m, 2 H, $CHCH_2SH$), 3.4 (m, 1 H, CH), 8.43 (bs, 3 H, NH_3^+). Anal. ($C_5H_{13}NS_2$·HCl) C, H, N.

Verification of the Optical Purity of Compound 22h. Saponification under N_2 of compound **21h** by degassed aqueous NaOH (1.1 equiv) yielded its sodium salt, Boc-NHCH($CH_2CH_2SO_3Na$)CH_2SNa. This compound was condensed with (*S*)-phenylbutyric acid chloride, obtained from 2(*S*)-phenylbutyric acid treated by $SOCl_2$ in refluxing CH_2Cl_2. The resulting phenylbutyryl thioester was checked on HPLC, showing a single peak (kromasil C8, 5 μm, 100 Å, A = H_2O–TFA 0.05%, B = CH_3CN, t_R 15.8 min, gradient 30%–60% B in 15 min), and a NMR TOXY experiment was carried out, confirming the presence of only one stereoisomer, which was therefore assumed to be the *S* isomer.

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